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(54) Title: TCF RESPONSIVE ELEMENT

(57) Abstract: Disclosed are DNA elements and constructs useful for obtaining tumour-selective gene expression in tumours having a mutated  $\beta$ -catenin/APC pathway. In particular, the use of these constructs to express genes encoding therapeutic proteins in colorectal cancer cells is described. The constructs comprise multiple repeats of a TCF-binding element operably linked to a promoter. By means of such a construct, tumour cell-specific expression of a prodrug-converting enzyme such as nitroreductase may be achieved. Coupled with systemic administration of a suitable prodrug, such as CB1954, selective killing of such tumour cells can be demonstrated.

## TCF RESPONSIVE ELEMENT

### BACKGROUND TO THE INVENTION

- 5 The present invention relates to a T cell factor (TCF)-responsive element, a gene and uses of the TCF-responsive element or nucleic acid construct in assays nucleic acid construct comprising a TCF-responsive element and a therapeutic and therapy.
- 10 TCFs are a family of transcription factors within the High Mobility Group (HMG) of DNA-binding proteins (Love *et al.*, Nature, 376, 791-795, 1995). The family includes TCF-1, TCF-3 and TCF-4 which are described in van der Wetering *et al.*, (EMBO J., 10, 123-132, 1991), EP-A-0 939 122 and Korinek *et al.* (Science, 275, 1784-1787, 1997). TCF-4 has been shown to be involved in tumorigenesis related
- 15 to Wnt/Wingless signalling. TCF and LEF-1 (lymphoid enhancer factor-1) are considered to mediate a nuclear response to Wnt signals by interacting with  $\beta$ -catenin. Wnt signalling and other cellular events that increase the stability of  $\beta$ -catenin are considered to result in transcriptional activation of genes by LEF-1 and TCF proteins in association with  $\beta$ -catenin. In the absence of Wnt signalling, LEF-
- 20 1/TCF proteins repress transcription in association with Groucho and CBP (CREB binding protein).

In the absence of Wnt signalling,  $\beta$ -catenin is found in two distinct multiprotein complexes. One complex, located at the plasma membrane, couples cadherins

25 (calcium dependent adhesion molecules) with the actin cytoskeleton whereas the other complex (containing the proteins adenomatous polyposis coli protein (APC), axin and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ )) targets  $\beta$ -catenin for degradation. Wnt signalling antagonises the APC-axin-GSK3 $\beta$  complex, resulting in an increase in the pool of free cytoplasmic  $\beta$ -catenin. The free cytoplasmic  $\beta$ -catenin can

30 translocate to the nucleus where it binds LEF-1/TCF factors and activates Wnt target genes. The regulation of LEF-1/TCF transcription factors by Wnt and other

signals is discussed in Eastman *et al*, (Current Opin. Cell Biology, 11, 233-240, 1999).

The APC gene is a tumour suppressor gene that is inactivated in most colorectal  
5 cancers. Mutations of APC are considered to cause the accumulation of free  $\beta$ -  
catenin, which then binds TCF causing increased transcriptional activation of  
genes including genes important for cell proliferation (e.g. cyclin D1 (Tetsu *et al.*,  
Nature 398, 422-426, 1999 and Shtutman *et al.*, PNAS USA, 96, 5522-5527,  
1999) and c-myc (He *et al.*, Science, 281, 1509-1512, 1998)). The involvement of  
10 APC in tumour development is discussed in He *et al*, (*supra*).

TCFs are known to recognise and bind TCF binding elements which have the  
nucleotide sequence CTTTGNN, wherein N indicates A or T (van der Wetering *et*  
*al*, *supra*).

15

TCF reporter genes have been constructed and are described in Korinek *et al*,  
(Science, 275, 1784-1787, 1997), Morin *et al*, (Science, 275, 1787-1790, 1997),  
EP-A- 0 939 122 and WO 98/41631. The TCF reporter gene is said to comprise  
three TCF binding elements upstream of either a minimal *c-Fos* promoter driving  
20 luciferase expression or a minimal herpes virus thymidine kinase promoter driving  
chloramphenicol acetyl-transferase expression. He *et al* (*supra*) discloses TCF  
reporter gene constructs comprising four TCF binding elements inserted into pBV-  
Luc.

25 There is a need for an effective treatment of cancers associated with a  
deregulation of the Wnt signalling pathway. Such cancers include most colorectal  
cancers, approximately 30% of melanomas and some breast, prostate and  
hepatocellular carcinomas.

30 There is also a need for a TCF response element which when linked to an  
expressible gene gives improved levels of expression and specificity.

## SUMMARY OF THE INVENTION

The present invention provides a nucleic acid construct comprising:

- 5 a TCF response element comprising:  
at least one TCF binding element having the sequence CTTTGNN,  
wherein N is A or T; and  
a promoter,  
and an expressible therapeutic gene operably linked to the TCF response  
10 element,  
wherein the TCF response element enables inducible expression of the operably  
linked therapeutic gene.

The term "inducible expression" as used herein means the level of expression  
15 obtained using the TCF response element is induced (i.e. increased) when one or  
more TCF/ $\beta$  catenin heterodimers binds to one or more of the TCF binding  
elements. Preferably the level of expression is increased by at least 15 fold, more  
preferably at least 25 fold and most preferably at least 30 fold.

20 The term "operably linked" as used herein refers to a *cis*-linkage in which the gene  
is subject to expression under control of the TCF response element.

The expressible gene comprises the necessary elements enabling gene  
expression when operably linked to the TCF response element, such as splice  
25 acceptor sequences, internal ribosome entry site sequences (IRES) and  
transcription stop sites. Such elements are well known to those skilled in the art.

It has been found that by using the nucleic acid construct of the present invention  
that expression of the operably linked therapeutic gene is only induced when  
30 TCF/ $\beta$  catenin heterodimers are present and capable of activating transcription.  
As cells that have become cancerous due to the deregulation of the Wnt signalling  
pathway have TCF/ $\beta$  catenin heterodimers, which activate transcription,

expression of the therapeutic gene will be induced. Accordingly, the nucleic acid construct of the present invention acts as a tumour selective promoter.

The nucleic acid construct of the present invention exhibits highly selective  
5 expression in that it gives no induction of expression of an operably linked gene above the background level in the absence of TCF/ $\beta$  catenin heterodimers or a functionally equivalent transcription activating factor.

The therapeutic gene can be any gene that on expression gives a therapeutic  
10 benefit. Preferred therapeutic genes include genes encoding toxins such as ricin and diphtheria toxin, and prodrug activating enzymes such as nitroreductases that activate CB1954, cytosine deaminase which activates 5-fluorocytosine, cytochrome P-450 which activates cyclophosphamide and paracetamol, and thymidine kinase which activates ganciclovir. Preferably the therapeutic gene  
15 encodes a nitroreductase. Suitable nitroreductases are described in EP-A-0638123 and Watanabe *et al*, (NAR, 18, 1059, 1990). Other preferred therapeutic gene include genes encoding immunomodulatory agents such as IL-2, IL-12, GM-CSF, B7-1 and B7-2 co-stimulatory molecules; genes encoding tumour suppressors such as RB, p53 and p16; and genes encoding apoptotic genes such  
20 as Bax, FasL and caspases.

The promoter can be any promoter that gives a desired level of expression of the operably linked gene. Suitable promoters include the SV40 promoter, the E1B promoter, and the *c-Fos* promoter. Preferably the promoter is the basal TATA box  
25 of the E1B promoter.

Preferably the TCF response element contains at least three and more preferably at least five TCF binding elements. It has been found that by using at least three and more preferably at least five TCF binding elements that an unexpected  
30 increase in expression can be obtained compared to a TCF response element containing fewer binding elements. This increase in expression is desirable for the production of a therapeutically effective amount of an encoded product.

Preferably the TCF response element comprises between 5 and 15 TCF binding elements, more preferably between 5 and 10 TCF binding elements and most preferably 5 TCF binding elements.

5

The TCF binding elements are preferably separated from each other by between 3 and 20 nucleotides, more preferably by between 3 and 14 and most preferably by between 10 and 12 nucleotides.

- 10 It is further preferred that the TCF binding elements are so spaced from each other as to be equally distributed radially around the DNA helix, especially when the promoter is the E1B promoter.

- It is preferred that the TCF binding element closest to the promoter is between 140  
15 and 10 nucleotides from the TATA box of the promoter. It is further preferred that the TCF binding element closest to the promoter is between 100 and 10 nucleotides, more preferably between 50 and 10 and most preferably between 30 and 15 nucleotides from the TATA box of the promoter.

- 20 In one preferred embodiment the TCF binding elements are separated from each other by between 3 or 4 nucleotides and the TCF binding element closest to the promoter is 25 nucleotides from the TATA box of the promoter.

The TCF binding elements preferably have the nucleotide sequence CTTTGAT.

25

The TCF binding elements can be in either orientation with respect to the promoter, namely 5' to 3' or 3' to 5'.

- The present invention also provides a nucleic acid construct designated herein as  
30 5merTCF-E1BTATA, which is shown schematically in Figure 6 and described in the materials and method section below.

The present invention also provides a TCF response element comprising:

at least five TCF binding elements; and

a promoter sequence,

wherein the TCF response element when operably linked to an expressible gene

5 gives inducible expression of the operably linked gene.

The TCF response element comprising at least five TCF binding elements can be used to obtain inducible expression of any operably linked gene such as a reporter gene or a therapeutic gene. Suitable reporter genes include luciferase,  $\beta$ -

10 galactosidase and chloramphenicol acetyl transferase.

The TCF response element comprising at least five TCF binding elements has been found to give improved (i.e. increased) levels of expression of an operably linked gene compared to a TCF response element comprising less than 5 TCF

15 binding elements.

The TCF binding elements and the promoter of the TCF response element comprising at least 5 TCF binding elements are as defined above.

20 The present invention also provides a TCF reporter construct comprising the TCF response element having at least 5 TCF binding elements operably linked to a reporter gene.

The present invention also provides the use of the TCF reporter construct of the present invention in a method of identifying candidate drugs for use in the treatment of cancers associated with the deregulation of the Wnt signalling pathway comprising the steps of:

contacting the TCF reporter construct with a test compound under conditions in which the reporter gene is transcribed; and

30 measuring the transcription of the reporter gene;

wherein a test compound which inhibits transcription of the reporter gene is a candidate drug for cancer treatment.

Preferably the step of contacting the TCF reporter construct is performed in the presence of a lysate from a cell with a deregulated Wnt signalling pathway.

- 5 The present invention also provides a vector comprising the nucleic acid construct of the present invention or the TCF responsive element having at least five TCF binding elements of the present invention operably linked to an expressible gene.

The vector may be any vector capable of transferring DNA to a cell. Preferably,  
10 the vector is an integrating vector or an episomal vector.

- Preferred integrating vectors include recombinant retroviral vectors. A recombinant retroviral vector will include DNA of at least a portion of a retroviral genome which portion is capable of infecting the target cells. The term "infection"  
15 is used to mean the process by which a virus transfers genetic material to its host or target cell. Preferably, the retrovirus used in the construction of a vector of the invention is also rendered replication-defective to remove the effect of viral replication of the target cells. In such cases, the replication-defective viral genome can be packaged by a helper virus in accordance with conventional techniques.
- 20 Generally, any retrovirus meeting the above criteria of infectiousness and capability of functional gene transfer can be employed in the practice of the invention.

- Suitable retroviral vectors include but are not limited to pLJ, pZip, pWe and pEM,  
25 well known to those of skill in the art. Suitable packaging virus lines for replication-defective retroviruses include, for example,  $\Psi$ Crip,  $\Psi$ Cre,  $\Psi$ 2 and  $\Psi$ Am.

- Other vectors useful in the present invention include adenovirus, adeno-associated virus, SV40 virus, vaccinia virus, HSV and poxvirus vectors. A  
30 preferred vector is the adenovirus. Adenovirus vectors are well known to those skilled in the art and have been used to deliver genes to numerous cell types, including airway epithelium, skeletal muscle, liver, brain and skin (Hitt, MM,



Addison CL and Graham, FL (1997) Human adenovirus vectors for gene transfer into mammalian cells. *Advances in Pharmacology*, 40: 137-206; and Anderson WF (1998) Human gene therapy. *Nature*, 392 : (6679 Suppl): 25-30).

- 5 A further preferred vector is the adeno-associated (AAV) vector. AAV vectors are well known to those skilled in the art and have been used to stably transduce human T-lymphocytes, fibroblasts, nasal polyp, skeletal muscle, brain, erythroid and haematopoietic stem cells for gene therapy applications (Philip *et al.*, 1994, *Mol. Cell. Biol.*, 14, 2411-2418; Russell *et al.*, 1994, *PNAS USA*, 91, 8915-8919; 10 Flotte *et al.*, 1993, *PNAS USA*, 90, 10613-10617; Walsh *et al.*, 1994, *PNAS USA*, 89, 7257-7261; Miller *et al.*, 1994, *PNAS USA*, 91, 10183-10187; Emerson, 1996, *Blood*, 87, 3082-3088). International Patent Application WO 91/18088 describes specific AAV based vectors.
- 15 Preferred episomal vectors include transient non-replicating episomal vectors and self-replicating episomal vectors with functions derived from viral origins of replication such as those from EBV, human papovavirus (BK) and BPV-1. Such integrating and episomal vectors are well known to those skilled in the art and are fully described in the body of literature well known to those skilled in the art. In 20 particular, suitable episomal vectors are described in WO98/07876.

Mammalian artificial chromosomes can also be used as vectors in the present invention. The use of mammalian artificial chromosomes is discussed by Calos (1996, *TIG*, 12, 463-466).

25

In a preferred embodiment, the vector of the present invention is a plasmid. The plasmid may be is a non-replicating, non-integrating plasmid.

- The term "plasmid" as used herein refers to any nucleic acid encoding an 30 expressible gene and includes linear or circular nucleic acids and double or single stranded nucleic acids. The nucleic acid can be DNA or RNA and may comprise

modified nucleotides or ribonucleotides, and may be chemically modified by such means as methylation or the inclusion of protecting groups or cap- or tail structures.

A non-replicating, non-integrating plasmid is a nucleic acid which when transfected  
5 into a host cell does not replicate and does not specifically integrate into the host cell's genome (i.e. does not integrate at high frequencies and does not integrate at specific sites).

Replicating plasmids can be identified using standard assays including the  
10 standard replication assay of Ustav *et al.*, EMBO J., 10, 449-457, 1991.

The present invention also provides a host cell transfected with the vector of the present invention. The host cell may be any mammalian cell. Preferably the host cell is a rodent or mammalian cell.

15

Numerous techniques are known and are useful according to the invention for delivering the vectors described herein to cells, including the use of nucleic acid condensing agents, electroporation, complexing with asbestos, polybrene, DEAE cellulose, Dextran, liposomes, cationic liposomes, lipopolyamines, polyornithine,  
20 particle bombardment and direct microinjection (reviewed by Kucherlapati and Skoultchi, *Crit. Rev. Biochem.* 16:349-379 (1984); Keown *et al.*, *Methods Enzymol.* 185:527 (1990)).

A vector of the invention may be delivered to a host cell non-specifically or  
25 specifically (i.e., to a designated subset of host cells) via a viral or non-viral means of delivery. Preferred delivery methods of viral origin include viral particle-producing packaging cell lines as transfection recipients for the vector of the present invention into which viral packaging signals have been engineered, such as those of adenovirus, herpes viruses and papovaviruses. Preferred non-  
30 viral based gene delivery means and methods may also be used in the invention and include direct naked nucleic acid injection, nucleic acid condensing peptides and non-peptides, cationic liposomes and encapsulation in liposomes.

The direct delivery of vector into tissue has been described and some short-term gene expression has been achieved. Direct delivery of vector into muscle (Wolff *et al.*, *Science*, 247, 1465-1468, 1990) thyroid (Sykes *et al.*, *Human Gene Ther.*, 5, 837-844, 1994) melanoma (Vile *et al.*, *Cancer Res.*, 53, 962-967, 1993), skin (Hengge *et al.*, *Nature Genet.*, 10, 161-166, 1995), liver (Hickman *et al.*, *Human Gene Therapy*, 5, 1477-1483, 1994) and after exposure of airway epithelium (Meyer *et al.*, *Gene Therapy*, 2, 450-460, 1995) is clearly described in the prior art.

- 10 Various peptides derived from the amino acid sequences of viral envelope proteins have been used in gene transfer when co-administered with polylysine DNA complexes (Plank *et al.*, *J. Biol. Chem.* 269:12918-12924 (1994)); Trubetskoy *et al.*, *Bioconjugate Chem.* 3:323-327 (1992); WO 91/17773; WO 92/19287; and Mack *et al.*, *Am. J. Med. Sci.* 307:138-143 (1994)) suggest that co-condensation of
- 15 polylysine conjugates with cationic lipids can lead to improvement in gene transfer efficiency. International Patent Application WO 95/02698 discloses the use of viral components to attempt to increase the efficiency of cationic lipid gene transfer.

- Nucleic acid condensing agents useful in the invention include spermine, spermine
- 20 derivatives, histones, cationic peptides, cationic non-peptides such as polyethyleneimine (PEI) and polylysine. 'Spermine derivatives' refers to analogues and derivatives of spermine and include compounds as set forth in International Patent Application WO 93/18759 (published September 30, 1993).

- 25 Disulphide bonds have been used to link the peptidic components of a delivery vehicle (Cotten *et al.*, *Meth. Enzymol.* 217:618-644 (1992)); see also, Trubetskoy *et al.* (*supra*).

- Delivery vehicles for delivery of DNA constructs to cells are known in the art and
- 30 include DNA/poly-cation complexes which are specific for a cell surface receptor, as described in, for example, Wu and Wu, *J. Biol. Chem.* 263:14621 (1988); Wilson *et al.*, *J. Biol. Chem.* 267:963-967 (1992); and U.S. Patent No. 5,166,320).

Delivery of a vector according to the invention is contemplated using nucleic acid condensing peptides. Nucleic acid condensing peptides, which are particularly useful for condensing the vector and delivering the vector to a cell, are described in International Patent Application WO 96/41606. Functional groups may be bound to peptides useful for delivery of a vector according to the invention, as described in WO 96/41606. These functional groups may include a ligand that targets a specific cell-type such as a monoclonal antibody, insulin, transferrin, asialoglycoprotein, or a sugar. The ligand thus may target cells in a non-specific manner or in a specific manner that is restricted with respect to cell type.

The functional groups also may comprise a lipid, such as palmitoyl, oleyl, or stearoyl; a neutral hydrophilic polymer such as polyethylene glycol (PEG), or polyvinylpyrrolidine (PVP); a fusogenic peptide such as the HA peptide of influenza virus; or a recombinase or an integrase. The functional group also may comprise an intracellular trafficking protein such as a nuclear localisation sequence (NLS), an endosome escape signal such as a membrane disruptive peptide, or a signal directing a protein directly to the cytoplasm.

The present invention also provides the nucleic acid construct, vector or host cell of the present invention for use in therapy.

Preferably, the nucleic acid construct, vector or host cell is used in the treatment of cancer.

25

The present invention also provides the use of the nucleic acid construct, vector or host cell of the present invention in the manufacture of a composition for use in the treatment of cancer.

30 The present invention also provides a method of treatment, comprising administering to a patient in need of such treatment an effective dose of the

nucleic acid construct, vector or host cell of the present invention. Preferably, the patient is suffering from cancer.

Preferably, the cancer is any cancer associated with the deregulation of the Wnt  
5 signalling pathway such as colorectal cancer, melanomas, breast, prostate and hepatocellular carcinomas.

The present invention also provides a pharmaceutical composition comprising the nucleic acid construct, vector or host cell of the present invention in combination  
10 with a pharmaceutically acceptable recipient.

The pharmaceutical compositions of the present invention may comprise the nucleic acid construct, vector or host cell of the present invention, if desired, in admixture with a pharmaceutically acceptable carrier or diluent, for therapy to treat  
15 a disease.

The nucleic acid construct, vector or host cell of the invention or the pharmaceutical composition may be administered via a route which includes systemic, intramuscular, subcutaneous, intradermal, intravenous, aerosol, oral  
20 (solid or liquid form), topical, ocular, as a suppository, intraperitoneal and/or intrathecal and local direct injection.

The exact dosage regime will, of course, need to be determined by individual clinicians for individual patients and this, in turn, will be controlled by the exact  
25 nature of the protein expressed by the therapeutic gene and the type of tissue that is being targeted for treatment.

The dosage also will depend upon the disease indication and the route of administration.  
30

The amount of nucleic acid construct or vector delivered for effective treatment according to the invention will preferably be in the range of between about 50 ng -

1000 µg of vector DNA/kg body weight; and more preferably in the range of between about 1-100 µg vector DNA/kg.

Although it is preferred according to the invention to administer the nucleic acid  
5 construct, vector or host cell to a mammal for *in vivo* cell uptake, an *ex vivo*  
approach may be utilised whereby cells are removed from an animal, transduced  
with the nucleic acid construct or vector, and then re-implanted into the animal.  
The liver, for example, can be accessed by an *ex vivo* approach by removing  
hepatocytes from an animal, transducing the hepatocytes *in vitro* and re-implanting  
10 the transduced hepatocytes into the animal (e.g., as described for rabbits by  
Chowdhury *et al.*, *Science* 254:1802-1805, 1991, or in humans by Wilson, *Hum.*  
*Gene Ther.* 3:179-222, 1992). Such methods also may be effective for delivery to  
various populations of cells in the circulatory or lymphatic systems, such as  
erythrocytes, T cells, B cells and haematopoietic stem cells.

15 The present invention also provides a composition for delivering the nucleic acid  
construct of the present invention or the TCF response element comprising at  
least 5 TCF binding elements of the present invention operably linked to an  
expressible gene to a cell.

20

## A BRIEF DESCRIPTION OF THE FIGURES

**Figure 1** shows the results of transient transfections of HeLa, HepG2 and  
SW480 cells with the Tcf responsive luciferase reporter construct "5merTcf-  
25 SV40-Luc" (CTL501) (Figure 1a). The numbers indicate the numbers of  
base pairs between the Tcf sites. The nucleotide sequence of 5merTCF-  
SV40 antisense strand is shown in Figure 1c. The sequences underlined  
and in italics are active TCF sites. The sequence just underlined is a  
mutated TCF site. The sequences in bold are the *Bgl*II, *Nhe*I and *Kpn*I  
30 recognition sites. Cells were transfected with equimolar amounts (about 1  
µg each) of the Tcf-responsive and control luciferase reporter constructs as  
indicated in Figure 1b. "SV40p" contains the SV40 promoter only; "SV40

e/p" contains both the SV40 promoter and enhancer; "CMV" contains the cytomegalovirus enhancer/promoter. Data are expressed as fold activation with respect to the activity of the SV40 promoter set as 1. The mean value and SD from two independent transfections are shown. This result is  
5 representative of three independent experiments.

**Figure 2** shows the quantitation of nitroreductase (NTR) expressed by HeLa, HepG2 and SW480 cells infected with CTL102 or with CTL501. Cells were infected at the indicated multiplicities of infection (moi, pfu/cell) with  
10 either CTL102, which expresses NTR from the CMV enhancer/promoter, c1 (CTL501), which contains the 5merTcf-SV40-NTR cassette in a left to right orientation or with c13, which contains the same cassette but in the right to left orientation. Cytoplasmic extracts were prepared two days later and assayed for NTR expression by ELISA (see materials and methods).  
15 Infections were done in duplicate. The mean NTR expression level from a representative experiment is shown.

**Figure 3** shows the antitumour efficacy of CTL501/CB1954 against HepG2 xenograft tumours in nude mice. Groups of six tumours ranging in size from  
20 20-80mm<sup>2</sup> cross sectional area were injected with approximately 10<sup>10</sup> viral particles as a single injection. Prodrug administration and tumour measurement are described in materials and methods.

**Figure 4** summarises the results of NTR immunostaining of subcutaneous  
25 SW480 xenografts in nude mice after a single injection of CTL501 or CTL102 (1.5 x 10<sup>10</sup> particles in 20 µl of 5% sucrose, 25mM Tris-HCl, pH 7.4). The tumours were excised after 48 hours and monitored for NTR expression as described in the materials and methods section. The average percentage NTR positive cells from 3 consecutive sections are shown for  
30 each dissected tumour.

**Figure 5** shows a comparison of the *in vivo* toxicity of CTL501 compared to CTL102 following systemic administration and prodrug treatment. Nude mice were intravenously injected (tail vein) with the indicated number of viral particles. After 48 hours CB1954 was given intraperitoneally for 5 consecutive days at 20 mg/kg body weight. The figure shows the maximum average weight loss (%) during the monitoring period (day 1-15) for each animal group (4 animals per group) and percentage of surviving animals for each treatment group after 15 days.

**Figure 6** shows the results of transient transfections of HeLa and SW480 cells with the Tcf responsive luciferase reporter construct 5merTcf-E1BTATA-Luc (CTL502) (Fig 6a). The nucleotide sequence of E1BTATA antisense strand is shown in Figure 6c. The sequence underlined and in italics is the E1B TATA box. The sequences in bold are the *HindIII*, *BglII*, *NheI* and *KpnI* recognition sites. Cells were transfected with equimolar (about 1 µg each) amounts of several luciferase reporter constructs as indicated in Figure 6b. pGL3basic contains a promoterless luc cDNA; "E1B" contains the Ad5 E1BTATA box upstream of the luc cDNA; 5merTcf-SV40 (CTL501) is described in Figure 1a. Data are expressed as fold activation compared to the activity of pGL3basic set as 1. The mean value and SD of duplicate transfections are shown. The data are representative of three independent experiments.

**Figure 7** shows, in schematic form, the four different arrangements of 5 Tcf binding elements that were evaluated in the work described in this document. For each construct the number of base pairs between the binding sites is shown, as well as the two dimensional arrangement of the sites along the DNA helix based on the assumption that 10.4 bp correspond to a complete turn of the helix.

30

**Figure 8** shows the results of transient transfections of SW480 cells using the indicated Tcf responsive luciferase reporter constructs. TcfA, TcfB and



TcfC were either combined with the minimal adenoviral E1BTATA box or with the SV40 basal promoter (see Figure 7 for a description of TcfA, TcfB and TcfC). The E1B, 5merTcf-SV40 (CTL501), 5merTcf-E1BTATA (CTL502) and CMV reporter plasmids are described in the legend to Figure

5 6. Cells were transfected with equimolar amounts (about 0.5 µg each) of each luciferase reporter constructs. Data are expressed as percentage activity of 5merTcf-E1BTATA (CTL502) (a) or 5merTcf-SV40 (CTL501) (b), respectively. The mean value and SD of triplicate transfections are shown. These data are representative of three independent experiments.

10

**Figure 9** shows the results of transient transfections of SW480 cells using Tcf-E1BTATA luciferase reporter constructs with different numbers and arrangements of Tcf binding sites. Figure 9a shows the number of Tcf sites and the spacing between them. Figure 9c shows the nucleotide sequence  
15 of the antisense strands of the Tcf-E1BTATA constructs. The sequences underlined and in italics are active Tcf sites. The sequences in bold are the *Bgl*II, *Nhe*I and *Kpn*I recognition sites. In the 4merTcf-E1BTATA construct the *Bgl*II site is defective. Cells were transfected with 0.5 µg of each luciferase reporter construct. Data are expressed as percentage activity of  
20 5merTcf-E1BTATA (CTL502) (Fig 9b). The mean value and SD of triplicate transfections are shown. The results are representative of three independent experiments.

**Figure 10** shows the results of transient transfections of SW480 cells with  
25 TcfC-E1BTATA-luc constructs with variable spacing between the proximal Tcf site and the TATA box (25-499 bp). The structure of the TcfC element is described in Figure 7. Figure 10a shows the structure of the TcfC-E1BTATA reporter constructs used for this assay. "d" indicates the number of base pairs from the last nucleotide of the proximal Tcf binding site to the first T  
30 of the TATA sequence. The 88 bp and 447 bp spacer fragments were derived by PCR from the human β-globin gene intron II. Cells were transfected with 0.5 µg of each luciferase reporter construct. Data are

expressed as percentage activity of the TcfC-E1BTATA d=25 construct (b). The mean value and SD of triplicate transfections are shown. The results are representative of three independent experiments. The nucleotide sequence of the antisense strand of TcfC-E1BTATA when d=25 is shown in Figure 10c. The sequences underlined and in italics are active Tcf sites. The sequences in bold are the E1B TATA box, and the *Sma*I and *Kpn*I recognition sites.

Figure 11 shows the quantitation by ELISA of NTR expressed by HeLa and SW480 cells infected with CTL102, CTL501 and CTL502. Cells were infected with a range of MOIs (1, 5, 20, 100, 500 and 1500 pfu/cell). Cytoplasmic extracts were prepared two days later and assayed for NTR by ELISA (materials and methods). Mean values of duplicate infections are shown.

Figure 12 shows a comparison of the levels of recombinant adenovirus-directed NTR expression in the livers of normal mice following i.v. injection with CTL102 and CTL501. Mice were tail vein injected with the indicated quantities of CTL102 or CTL501, sacrificed 48 h later and livers stained for NTR as described in Materials and methods for tumour sections.

Figure 13 shows a comparison of the level of anti-tumour efficacy of CTL501/CB1954 with CTL102/CB1954 in a xenograft model of colon cancer. Groups of SW480 tumours (n=5) ranging in size from 20-80mm<sup>2</sup> cross sectional area were injected with a single dose (10<sup>9</sup> or 10<sup>10</sup> particles) of either CTL102 or CTL501. Prodrug treatment and tumour measurement were done as described in Materials and methods.

Figure 14 shows a comparison of the level of anti-tumour efficacy of CTL503/CB1954 with CTL102/CB1954 in a xenograft model of colon cancer. Groups of SW480 tumours (n=5) ranging in size from 20-80mm<sup>2</sup> cross sectional area were injected with a single dose (10<sup>10</sup> particles) of either CTL102 or CTL503.

Prodrug treatment and tumour measurement were done as described in Materials and methods.

**Figure 15** shows that CTP1 and CTP3 express at very low levels in normal human  
5 endothelial cells, dermal fibroblasts and hepatocytes. Cells were infected with  
Ad.CMV-nLacZ, Ad.CTP1-nLacZ and Ad.CTP3-nLacZ as described in Materials  
and methods and analysed 48 h later. For the data shown in Figure 15a and b,  
extracts were prepared from cells infected with the indicated MOIs (pfu/cell) and  
beta-galactosidase assayed using the Galacto-Light assay system as described by  
10 the supplier. Error bars represent standard deviation. A representative experiment  
is shown in each case. In Figure 15c, cells were infected with an MOI of 1000  
pfu/cell and beta-galactosidase-expressing cells visualised by X-Gal staining. Only  
fibroblasts infected with Ad.CMV-nLacZ expressed detectable enzyme. Figure 15d  
shows that all three viruses directed high-level beta-galactosidase expression in  
15 infected SW480 colon cancer cells.

**Figure 16** shows that CTP1 and CTP3 express at very low levels under replicating  
conditions in the adenovirus helper cell lines (PerC6 and 293). PerC6 and 293  
cells were infected at an MOI of 100 and harvested 30 h later. Cell extracts were  
20 then prepared and assayed for  $\beta$ -galactosidase as described in Materials and  
methods. Error bars represent standard deviation. A representative experiment is  
shown. Figure 16a has a logarithmic, and 16b a linear, scale.

**Figure 17** shows that CTP1 and CTP3 express at high levels in secondary  
25 colorectal cancer tissue but not in attached liver tissue. 2-3mm<sup>3</sup> segments of  
freshly excised secondary colorectal cancer (liver) with attached liver margin  
tissue were incubated in the indicated viruses, fixed 48 h later and stained for  $\beta$ -  
galactosidase expression as described in Materials and methods. "T" denotes  
tumour tissue, "L" denotes attached liver tissue.

**Figure 18** shows that high-level CTP1 and CTP3 expression in primary colorectal cancer specimens correlates with high-level, non-membranous expression of  $\beta$ -catenin.

2-3mm<sup>3</sup> segments of freshly excised primary colorectal cancer were transduced with the indicated viruses and stained for  $\beta$ -galactosidase expression as described in Materials and methods. Samples of each tumour were sectioned and stained for  $\beta$ -catenin expression as described in Materials and Methods.

## 10 DETAILED DESCRIPTION OF THE INVENTION

The invention is described in detail by the use of the following examples. These are by way of illustration only and are not to be taken as limiting.

## 15 EXAMPLES

### Materials and methods

#### *Cell culture*

20 HepG2 (human liver carcinoma; mutated  $\beta$ -catenin), SW480 (human colon carcinoma, mutated APC) and HeLa (human cervix carcinoma) cell lines were obtained from ATCC and were maintained as recommended by the supplier. PER.C6 (human embryonic retinoblast cell line) cells were obtained from IntroGene, (Fallaux et al., Human Gene Ther., 9: 1909-1917, 1998) and were  
25 cultured in DMEM supplemented with 10% FCS, 2 mM MgCl<sub>2</sub> and antibiotics (150  $\mu$ g/ml penicillin, 250  $\mu$ g/ml streptomycin).

Primary human dermal fibroblasts were isolated from punch biopsy samples from healthy volunteers and maintained as above. HUVEC cells were obtained from Promocell (Heidelberg, Germany) and maintained in Endothelial Growth Medium plus Supplement Mix (Promocell). Primary hepatocytes were maintained in  
30 Williams medium with added antibiotics, glutamine, insulin and hydrocortisone.

*Plasmid construction*

To clone pGL3pro/5merTcf-SV40 (CTL501) two partially double-stranded fragments were generated by annealing the partly complementary pairs of

5 oligonucleotides 1 with 2, and 3 with 4.

Oligo 1: PCT AGC AAG CTT ACT AGT CCT TTG ATC AAG AGT CCT ACC TTT  
GAT CTC TAA ATG CAC CTT TGA TC

10 Oligo 2: PAC TGA ATT CCT TGA TCA AAG GTG CAT TTA GAG ATC AAA GGT  
AGG ACT CTT GAT CAA AGG ACT AGT AAG CTT G

Oligo 3: PAA GGA ATT CAG TCC TTT GAT CAA GAG TCC TAC CTT TGA TCT  
CTA AAT GCA CCT TTG ATC A

15

Oligo 4: PGA TCT GAT CAA AGG TGC ATT TAG AGA TCA AAG GTA GGA CTC  
TTG ATC AAA GG

(where P indicates phosphate modification)

20 These fragments each contain three TCF binding sites (consensus  
CCTTTGATC). These two double-stranded fragments were ligated and the  
resulting fragment (about 130 bp) containing six TCF sites was cloned by their  
*NheI* and *BglII* sites into *NheI/BglII*-digested pGL3 promoter plasmid (pGL3pro;  
Promega) resulting in pGL3pro/5merTcf-SV40 clone 10. However, sequencing  
25 revealed that the most 3' TCF-binding site contained a deletion of one G  
nucleotide, producing an inactive site (CCTTTATC) (See Figure 1c).

To generate pGL3basic/5merTcf-E1BTATA (CTL502) 84 bp from the Clontech  
plasmid pG5CAT, spanning 21 bp upstream and 55 bp downstream of the  
30 adenoviral E1BTATA box (TATATAAT), were amplified with PCR using oligos  
containing *BglII* and *HindIII* overhangs. This fragment, containing 16 bp of the Ad5  
E1B promoter (GGGTATATAATGCGCC), was then cloned into *BglII/HindIII* -

digested pGL3basic resulting in pGL3basic/E1BTATA clone 2 (see Figure 6c). Then the 5-mer TCF sites from pGL3basic/5merTcf-SV40 (CTL501) clone 10 were cut out by *NheI/BglII* digestion and cloned into *NheI/BglII*-digested pGL3basic/E1BTATA clone 2, resulting in pGL3basic/5merTcf-E1BTATA clone 1.

5 Sequencing confirmed the expected construct.

To generate pGL3pro/TcfA-SV40 clone 4, pGL3pro/TcfB-SV40 clone 31, pGL3pro/TcfC-SV40 clone 3, pGL3basic/TcfA-E1BTATA clone 28, pGL3basic/TcfB-E1BTATA clone 10 and pGL3basic/TcfC-E1BTATA clone 1, oligonucleotides

10 5, 7 and 9, respectively, each containing 5 active TCF binding sites (CCTTTGATC) and an intentionally mutated site (CCTTTATC, for consistency) were annealed to their respective antisense oligonucleotides, 6, 8 and 10.

Oligo 5: CTA GCA AGC TTA CTA GTC CTT TGA TCA AGA GTT TCC TAC CTT

15 TGA TCT CTA AAT TGC ACC TTT GAT CAA GGA ATT CAG TCC TTT GAT CAA  
GAG TAA CCT ACC TTT GAT CTC TAA ATG CAC CTT TAT CA

Oligo 6: GAT CTG ATA AAG GTG CAT TTA GAG ATC AAA GGT AGG TTA CTC  
TTG ATC AAA GGA CTG AAT TCC TTG ATC AAA GGT GCA ATT TAG AGA

20 TCA AAG GTA GGA AAC TCT TGA TCA AAG GAC TAG TAA GCT TG

Oligo 7: CTA GCA AGC TTA CTA GTC CTT TGA TCA AGC TAC CTT TGA TCT  
CTA GCA CCT TTG ATC AAG AGT CCT TTG ATC AAG CCT ACC TTT GAT  
CTC TAA ATG CAC CTT TAT CA

25

Oligo 8: GAT CTG ATA AAG GTG CAT TTA GAG ATC AAA GGT AGG CTT GAT  
CAA AGG ACT CTT GAT CAA AGG TGC TAG AGA TCA AAG GTA GCT TGA  
TCA AAG GAC TAG TAA GCT TG

30 Oligo 9: CTA GCA AGC TTA CTA GTC CTT TGA TCA ATA CCT TTG ATC TCA  
CCT TTG ATC AAG TCC TTT GAT CAT ACC TTT GAT CTC TAA ATG CAC CTT  
TAT CA

Oligo 10: GAT CTG ATA AAG GTG CAT TTA GAG ATC AAA GGT ATG ATC  
 AAA GGA CTT GAT CAA AGG TGA GAT CAA AGG TAT TGA TCA AAG GAC  
 TAG TAA GCT TG

5

(all six oligonucleotides were modified by 5' phosphate).

The resulting fragments containing *NheI/BglII* overhangs were cloned into  
*NheI/BglII* digested pGL3promoter or *NheI/BglII* digested pGL3basic/E1BTATA  
 10 clone 2 to generate the aforementioned constructs. The sequences were  
 confirmed to be as expected.

The constructs pGL3basic/2merTcf-E1BTATA clone 9, pGL3basic/3merTcf-  
 E1BTATA clone 2, pGL3basic/3merTcf-E1BTATA clone 13, pGL3basic/4merTcf-  
 15 E1BTATA clone 15 and pGL3basic/4merTcf-E1BTATA clone 34 were cloned by  
 the same way, but, these constructs have one or more Tcf sites deleted most  
 probably due to loop generation during annealing and following excision of loops in  
*E. coli* after transformation. For spacing between the TCF sites and confirmed  
 sequences for these constructs see Figures 9a and c.

20

pGL3basic/88-E1BTATA clone 8 and pGL3basic/TcfC-88-E1BTATA clone 2  
 (distance to TATA box: d=140) were constructed by cloning a PCR amplified 88 bp  
 fragment (5' oligo: GAAGATCTCCCCTTCTTTTCTATGGTTAAG, 3' oligo:  
 GAAGATCTGCAATCATTCTGTCTGTTTCCC) from the human  $\beta$ -globin gene intron  
 25 II using *BglII* overhangs into *BglII* -digested pGL3basic/E1BTATA clone 2 or *BglII*  
 digested pGL3basic/TcfC-E1BTATA clone 1, respectively.

pGL3basic/447-E1BTATA clone 1 and pGL3basic/TcfC-447-E1BTATA clone 6  
 (distance to TATA box: d=499) were cloned by inserting a PCR amplified 447 bp  
 fragment (5' oligo: GAAGATCTCCCCTTCTTTTCTATGGTTAAG, 3' oligo:  
 30 GAAGATCTGATTTGG.TCAATATGTGTACAC) from the human  $\beta$ -globin gene  
 intron II using *BglII* overhangs into *BglII* -digested pGL3basic/E1BTATA clone 2 or

*Bgl*II -digested pGL3basic/TcfC-E1BTATA clone 1. The sequences were confirmed.

To create pGL3basic/TcfC-25-E1BTATA clone 6 (distance to TATA box: d=25),  
5 the TcfC fragment was cut out by *Hind*III/*Bgl*II digest from pGL3basic/TcfC-E1BTATA clone 1 and then blunted with mung bean nuclease (NEB). The blunted fragment was cloned into a partially *Xba*I -digested, mung bean nuclease-blunted pGL3basic/E1BTATA clone 2, to create the intermediate construct pGL3basic/TcfC-25-E1BTATA clone 19. From here, we had to reclone the TcfC-  
10 25-E1BTATA promoter fragment into pGL3basic as the plasmid backbone immediately upstream of the promoter was changed for unknown reasons in clone 19. Therefore the TcfC-25-E1BTATA promoter was cut out by *Spe*I/*Hind*III digest from pGL3basic/TcfC-25-E1BTATA clone 19 and then blunted with Klenow enzyme. Finally, the blunted TcfC-25-E1BTATA fragment was cloned into *Hind*III  
15 digested and Klenow-blunted pGL3basic to generate pGL3basic/TcfC-25-E1BTATA clone 6 (d=25) with the correct plasmid backbone. The sequence was determined since the blunting process is error-prone (see Fig 10c)

#### *Transient transfections and luciferase assay*

20 HepG2, SW480 and HeLa were seeded the day before transfection at densities of  $2.5 \times 10^5$ ,  $1.5 \times 10^5$  and  $6.0 \times 10^4$  cells per 6-well respectively. The next day, a mixture of CL22 peptide (KKKKKKGGFLGFWRGENGRKTRSAYERMCNILKGK (described in International Patent Application WO 98/35984)) and plasmid DNA (2:1 ratio,  $\mu\text{g}:\mu\text{g}$ ) was  
25 prepared in a final volume of 100  $\mu\text{l}$  in HBS (10 mM Hepes pH 7.4, 150 mM NaCl; Sigma) and incubated at RT for 30-45 min before addition of 0.9 ml of "RAC" solution (0.1% human albumin (BPL, UK), 120  $\mu\text{M}$  chloroquine (Sigma) in RPMI medium (Sigma). The transfection solution was then added to cells after washing them once with PBS and incubated with the cells for 4-5 hours before replacement  
30 with 2ml of fresh complete medium. After two days, cells were washed once with PBS and then incubated for 10 min at RT in 200  $\mu\text{l}$  lysis buffer (10 mM sodium phosphate pH 7.8, 8 mM  $\text{MgCl}_2$ , 1 mM EDTA pH 8.0, 1% Triton X-100 and 15%



glycerol). After centrifugation, an aliquot of the supernatant was assayed for luciferase activity with luciferase assay buffer (0.1 mM luciferin, 0.44 mM ATP in lysis buffer) using a luminometer (Lumat LB 9501, Berthold, Germany). Activity was normalised using the protein content of each sample (BCA, Pierce).

5

*Construction of replication defective Adenoviruses expressing NTR*

The transfer vectors used to construct the recombinant adenoviruses c1/CTL501, c13 and CTL502 i.e. pPS1128/5merTcf-SV40 (clones 1 and 13) and pPS1128/5merTcf-E1BTATA (clone 10) respectively were constructed in two  
10 stages. In the first, the 5merTcf-SV40 promoter from pGL3pro/5merTcf-SV40 clone 10 and the 5merTcf-E1BTATA promoter from pGL3basic/5merTcf-E1BTATA clone 1 were cloned as Hind III fragments into HindIII digested pTX0374 resulting in replacement of the CMV enhancer/promoter with the respective Tcf promoters to create "pTX0374/5merTcf-SV40 clone 8" and "pTX0374/5merTcf-E1BTATA  
15 clone 1". pTX0374 contains a CMV-NTR-IVSII-p(A) (NTR: *E. coli* B/r nitroreductase gene amplified from genomic DNA) expression cassette with the human  $\beta$ -globin intron II for transcriptional stabilisation and the Complement 2 gene poly(A) signal for termination and the plasmid pBluescript KS+ as backbone. In the second stage, the complete expression cassettes from pTX0374/5merTcf-SV40 clone 8 and pTX0374/5merTcf-E1BTATA clone 1 were recloned into SpeI  
20 digested pPS1128 to create "pPS1128/5merTcf-SV40 clones 1 and 13" (clone 1: left to right orientation in E1; clone 13: right to left orientation in E1) and "pPS1128/5merTcf-E1BTATA clone 10 (left to right orientation in E1). pPS1128 was kindly provided by Dr. P. Searle, CRC Institute of Cancer Studies, University  
25 of Birmingham. pPS1128 contains adenoviral sequences from the left hand ITR to nt 359 and from nt 3525 to 10,589 and is therefore an E1-deleted vector.

pTX0375, the transfer vector used to generate CTL102, was constructed by cloning a SpeI fragment spanning the whole expression cassette (CMT-NTR-  
30 IVSII-p(A)) from pTX0374 into SpeI digested pPS1128 and identification of a clone containing the cassette in the left to right orientation.

The adenoviral "backbone" vector pPS1160 was constructed by *PacI* linearisation of pPS1128, ligation with a *PacI*-compatible adaptor (oligo1: 5'-TACATCTAGATAAT-3', oligo2: 5'-TTATCTAGATGTA-3') containing an *XbaI* site followed by *XbaI* digestion to release a ca. 7kb *XbaI* fragment containing Ad5 sequences 3524-10589. This was then cloned into *XbaI* linearised pPS1022 (Dr. Peter Searle) a pUC18-based plasmid containing Ad5 sequences from nt 10,589 to the right hand ITR but lacking nt 28,592 to 30,470 (E3 region).

The recombinant viruses CTL501, CTL502 and CTL102 were constructed by homologous recombination in Per.C6 cells. These were cotransfected with an equimolar mixture of pPS1128/5merTcf-SV40 (clone 1 or clone 13), pPS1128/5merTcf-E1BTATA (clone 10) or pTX0375, respectively, and pPS1160 into 90% confluent PER.C6 cells. The recombinant viruses were harvested about 7 days later by 3 freeze-thaw cycles in infection medium (DMEM, 1% FCS, 2 mM MgCl<sub>2</sub>). By repeated infection/harvesting cycles the viruses were grown to large scale and then purified by standard CsCl density centrifugation, dialysed against excess of storage buffer (10 mM Tris pH 7.4, 140 mM NaCl, 5 mM KCl, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.9 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub> and 5% sucrose) and finally snap-frozen in liquid nitrogen and stored at -80°C. Particle concentrations were determined using the BCA Protein Assay Reagent (Pierce). Infectious titres were either estimated on the assumption that 1 in 100 particles are infectious or were determined in a limiting dilution standard method against a defined internal virus standard. Adenoviral DNA was characterised by restriction digestion and direct sequencing using viral DNA as a template (the promoter region up to the starting NTR reading frame was sequenced).

pTX0374 was constructed by cloning a 1.6kb *BglII*-*BamHI* fragment containing the CMV promoter fused to the *E. coli* ntr gene into pSW107. This plasmid was constructed by cloning a 917bp fragment of the human beta-globin gene (*BamHI* site in exon2 to the *EcoRI* site in exon3) coupled to a 240bp *HincII*-*BamHI* fragment containing the polyA addition and transcriptional termination signals of the human complement C2 gene into pBluescript (Stratagene). pTX0375 was

constructed by cloning a 2.5kb *SpeI* fragment from pTX0374 into *SpeI*-digested pPS1128. This plasmid was constructed in two stages. In the first, the left hand *EcoRI* site of pPS971 (Weedon *et al*, Int. J. Cancer, in press) was converted to a *SwaI* site to create pPS115. In the second, the 350bp *SpeI*-*AflIII* fragment of

5 pPS115 was replaced with a linker prepared by annealing the two oligonucleotides:

5'- CTAGTATCGATTGTTAATTAAGGGCGTGGCC -3' and

5'- TTAAGGCCACGCCCTTAATTAACAATCGATA -3'.

pPS1022 was constructed from pPS972 by conversion of the right hand *EcoRI* site

10 to a *SwaI* site.

It will be appreciated by those skilled in the art that any suitable vector may be used in the construction of the Tcf responsive element of the present invention. In particular, it will be appreciated that any suitable adenovirus based vector can be

15 used in the construction of the Tcf responsive element of the present invention.

#### *Preparation of adenoviral DNA*

Viral DNA was prepared from about  $10^{11}$  CsCl-banded virus particles by incubation with 100 µg/ml proteinase K in 20 mM Tris/HCl pH7.5, 5 mM EDTA pH8.0, 0.1% SDS for 3-4 hours at 37°C. The crude DNA preparation was then extracted two

20 times with an equal volume of Phenol:Chloroform:Isoamylalcohol (25:24:1), once with chloroform only and then precipitated with 1/10 Vol. 3.0 M Na Acetate and 2 Vol. 100% Ethanol in a dry ice/ethanol bath for 10 min. After centrifugation, (10 min at 13K at RT) the resultant DNA pellet was washed with 70% Ethanol, air-

25 dried and then resuspended in water. Viral DNA was analysed by restriction digestion. The promoter regions of CTL501 and CTL502 were analysed by DNA sequencing (Seqlab GmbH, Germany).

#### *Assay of NTR expressed by virus transduced cells (ELISA)*

30  $1-1.5 \times 10^4$  cells per 6-well cell culture plate were transduced by incubation with virus in infection medium (DMEM/1% FCS) for 90 min at 37°C in a 5% CO<sub>2</sub> atmosphere followed by incubation in complete medium for 2 days. Cytoplasmic

extracts were then prepared by hypotonic lysis. Cells were washed with PBS before lysis with ice-cold hypotonic lysis buffer (10 mM Tris pH 7.5) for 45 min on ice. Extracts were cleared by centrifugation at 13K for 2 min. 96-wells (Nunc-Immuno Plate Maxisorp Assay Plates) were coated overnight in triplicate with 50  $\mu$ l extract. Wells were washed three times with PBS/0.5% Tween20 and then incubated with sheep anti-NTR polyclonal antibody (100  $\mu$ l of 1:2,000 dilution in PBS/0.5% Tween20) for 30 min at RT. After removal of excess primary antiserum by washing 3x with PBS/0.5% Tween20 the extracts were incubated with donkey anti-sheep HRP conjugate (100  $\mu$ l of 1:5,000 dilution in PBS/0.5% Tween20) for 30 min at RT. After three washes with PBS wells were incubated with 100  $\mu$ l of a solution prepared by mixing 1 ml of TMB (1 mg/ml in DMSO; Sigma) with 9 ml 0.05 M phosphate-citrate buffer (Sigma) and 2  $\mu$ l of 30% (v/v)  $H_2O_2$ . Wells were incubated for 15 min at RT and the reaction stopped with 25  $\mu$ l of 2M  $H_2SO_4$ . OD 450nm was read using a 96-well plate reader (Labsystems Multiscan MS).

#### *NTR immunostaining of virus-injected xenografts*

SW480 tumours were injected with 20  $\mu$ l of either CTL102 or CTL501 and excised 48 hours later following humane sacrifice. The tumours were then fixed in 4% formalin/PBS for 20-24 hours at 4°C before embedding in paraffin wax (Citadel 2000). 3-4 $\mu$ m wax sections were cut and collected on APES-treated glass slides. Sections were dewaxed, rehydrated and washed 2x in PBS/0.01% Tween20 (5 min) and then immersed in 0.25%  $H_2O_2$ /PBS for 30 min at RT. Sections were then washed 3x in PBS/0.01% Tween20 (5min) and then permeabilised with ice cold 0.1% Triton-X100 for 5 min at RT followed by 2x washing for 5 min in PBS/0.01% Tween20. Sections were then blocked with 5% normal rabbit serum in PBS for 60 min at RT before incubation with a polyclonal sheep anti-NTR (1:2,000 diluted in PBS) for 60 min at RT. Excess primary antibody was removed by washing 3x with PBS/0.01% Tween20 (5 min) before incubation with a biotinylated anti-sheep IgG/Streptavidin-HRP solution (Vectastain ABC kit, Vector Laboratories, PK-6106, 1:200 diluted in PBS) for 30 min at RT.

Sections were washed 3x for 5 min with PBS/0.01% Tween20 and then incubated with freshly prepared AEC reagent (Vectastain, Vector Laboratories). Reactions were stopped after 10 min by washing in water and finally sections were mounted in an aqueous mountant. Staining was analysed by counting the number of  
5 positively stained cells within a standardised area.

*Generation of subcutaneous tumour xenografts in nude mice*

Tumour xenografts were generated by subcutaneous injection of one flank of male Balb/c nu/nu mice (6-8 weeks old, Harlan UK) with 100 µl of a  
10 suspension of exponentially growing cultured tumour cells, washed and resuspended in sterile saline solution. Cell viability was at least 90%. For HepG2 an inoculum of  $5 \times 10^6$  cells was used. For SW480 this was slightly lower at  $2 \times 10^6$  cells. Following injection, mice were kept in a sterile environment and examined regularly for the appearance of tumour  
15 xenografts.

*Intratumoural injection of CTL102 and CTL501*

A U-100 insulin syringe (TERUMO, Leuven, Belgium) fitted with a fixed 27-gauge needle was used to inject 20 µl of virus suspension or vehicle alone  
20 (5% sucrose in 25mM Tris-HCl, pH 7.4) directly into tumours through the skin. To avoid virus leakage, injections were performed in a continuous slow movement and the needle was held in place for about 15 seconds after injection was completed.

25 *CB1954 treatment schedule and tumour size measurements*

CB1954, freshly dissolved in DMSO and diluted 1 in 5 with NSS, was administered by intraperitoneal injection. Mice received 5 consecutive daily doses of 20 mg/kg body weight. Mice treated with the vehicle alone were injected with 20% DMSO/saline (5.0 µl/kg body weight). Tumour growth  
30 was monitored by measuring the tumour diameter through the skin in two perpendicular dimensions (length and width) using callipers and expressed as surface area (length x width = mm<sup>2</sup>). To prevent undue suffering

compulsory sacrifice was carried out when the tumour reached 140 mm<sup>2</sup>. Weight loss and changes in animal behaviour (signs of distress) were also recorded.

5 *Intravenous injection of CTL501/CTL102 and CB1954 administration in nude mice*

A syringe fitted with a fixed 27-gauge needle was used to administer 100 µl of virus suspension into the tail vein of nude mice. After 48 hours, CB1954 was administered as described above. Mice were monitored and weighed  
10 daily. To prevent undue suffering to animals humane sacrifice was carried out if mouse body weight was reduced by more than 20% or at the onset of any sign of severe distress.

*Ex vivo transduction of freshly excised colorectal tumour samples*

15 Freshly excised tumour tissue was extensively washed (at least 10 minutes duration) under aseptic conditions with 20 ml of Earl's MEM containing 10% FCS, 150 µg/ml penicillin, 250 µg/ml streptomycin, 10 µg/ml tetracycline, 100 µg/ml amikacin, 150 g/ml chloramphenicol and 100 µg/ml gentamycin and stored at 4°C overnight in medium containing 10% FCS. After removal of fat and grossly and  
20 suspected necrotic tissue, 2-3 mm<sup>3</sup> samples were prepared and placed individually into wells of a 96-well plate. Samples (in quadruplicate) were incubated in 150 µl of serum-free medium containing,  $1.0 \times 10^{10}$  virus particles or in medium alone for 4 h in a CO<sub>2</sub> incubator. The medium was then replaced with EMEM containing 10% FCS and the samples incubated for a further 44h to allow gene expression to  
25 proceed. β-galactosidase expression was visualised after fixation of the tumour samples in 2% paraformaldehyde/PBS for 2 h at 4°C and washing with PBS by overnight incubation in X-gal staining solution at 37°C.

*β-catenin immunohistochemical staining*

30 Paraffin-embedded sections were stained for β-catenin using rabbit polyclonal antiserum (Santa Cruz Biotechnology Inc) and vector AEC kit.

*Construction of CTL503, Ad.CTP1-nLacZ and Ad.CTP3-nLacZ*

The transfer vector for CTL503 was constructed by cloning the TCFC<sub>d=25</sub>-E1BTATA promoter as a *HindIII* fragment upstream of the NTR gene in *HindIII*-digested pTX0374, so removing the CMV promoter from the latter. The new expression cassette was cloned into pPS1128 as a *SpeI* fragment to create pPS1128/TCFC<sub>d=25</sub>-E1BTATA.

The transfer vector for Ad.CTP1-nLacZ was constructed by cloning the an expression cassette comprising of a nuclear-targeted LacZ gene fused to the mouse protamine polyadenylation signal as an *XbaI* fragment into *XmaI/SpeI*-digested and blunted pTX0374. The CTP1 promoter was cloned upstream of the nLacZ gene as a *HindIII* fragment. The CTP1-nLacZ expression cassette was then cloned in a left-to-right orientation into pPS1128 as a blunted, *SpeI/NotI* fragment into *SpeI*, blunted pPS1128 to create pPS1128/CTP1-nLacZ.

To construct the transfer vector for Ad.CTP3-nLacZ, in a first step the CTP3 promoter was cloned as a blunted, *SpeI/HindIII* fragment into *HindIII*-digested and blunted pTX0374, replacing the CMV promoter with CTP3. This cloning regenerated the *HindIII* site. In a second step, the resulting plasmid was digested with *HindIII/PacI* and then blunted to release the NTR gene and the IVSII intron/polyadenylation signal. The nLacZ-poly(A) cassette (see details described for the construction of CTP1-nLacZ) was cloned downstream of CTP3 as a blunted *XbaI* fragment. Finally the complete expression cassette was cloned into pPS1128 in a left-to-right orientation as a blunted, *SpeI/NotI* fragment into the *PmeI* site of a pPS1128 derivative (pTX0398) in which the unique *SpeI* site is replaced by a *PmeI* site to create pTX0398/CTP3-nLacZ.

Viruses were rescued by homologous recombination of the above transfer vectors with pPS1160 in PerC6 cells as described above (*Construction of replication defective adenoviruses expressing NTR*)

*X-gal staining of cells for histology*

After washing cells with PBS, cells were fixed in 0.05% glutaraldehyde in PBS for 10 min at RT. Following further washing in PBS, cells were incubated cells the following solution and incubated at 37°C. X-gal solution: 400  $\mu$ l 500 mM

$K_4Fe(CN)_6$ , 400  $\mu$ l 500 mM  $K_3Fe(CN)_6$ , 100  $\mu$ l 2mM  $MgCl_2$ , 250  $\mu$ l 40 mg/ml X-Gal in DMF and 8.85 ml PBS.

*Quantitation of  $\beta$ -galactosidase expression*

- 5 Expression of  $\beta$ -galactosidase was measured for quantitative graphical presentation by means of the Galacto-Light system (Tropix Inc, Applied Biosystems, Foster City, CA, U.S.A.)

10 **Examples**

**Example 1**

*A 5merTcf-SV40-Luciferase construct is specifically activated in tumour cell lines with deregulated  $\beta$ -catenin activity.*

- 15 To evaluate the ability of  $\beta$ -catenin/Tcf binding elements to direct high-level gene expression specifically in cells with deregulated  $\beta$ -catenin activity we constructed a luciferase reporter plasmid containing an artificial promoter comprising of 5 Tcf sites upstream of the basal SV40 promoter. In HeLa cells ( $\beta$ -catenin not deregulated) the 5merTcf-SV40 was not more active than the SV40 promoter  
20 alone. In contrast, in cell lines with deregulated  $\beta$ -catenin the 5merTcf-SV40 promoter expressed at about 80% activity of the CMV enhancer/promoter (SW480 cells) and was even more active than CMV (HepG2). The induction ratios for the 5merTcf-SV40 were 18 (HepG2) and 44.2 (SW480). These data indicate that the Tcf sites are active only in the presence of nuclear  $\beta$ -catenin. In some experiments  
25 the activity of the 5merTcf-SV40 promoter in Hela cells was even lower than the activity of SV40 alone (data not shown). This is consistent with the fact that Tcf factors normally repress transcription e.g. by interacting with the transcriptional cofactor CBP (cAMP binding protein) if  $\beta$ -catenin is not present to create  $\beta$ -catenin/Tcf heterodimers.



**Example 2**

*The activity and specificity of the 5merTcf-SV40 artificial promoter is retained in a replication defective adenovirus vector.*

To evaluate the utility of the 5merTcf-SV40 promoter to drive the high-level  
5 expression of a therapeutic gene selectively in tumours comprising of cells with  
deregulated  $\beta$ -catenin, for instance a tumour of colorectal origin, we constructed a  
replication defective Adenovirus vectors expressing the *E. coli* B nitroreductase  
gene (NTR) under the control of the 5merTcf-SV40 promoter. The NTR gene  
encodes an enzyme that can convert the prodrug CB1954 into a potent DNA  
10 cross-linking agent that can kill both dividing and non-dividing cells. Clones 1  
(CTL501) and 13 contain the cassette in the indicated orientation (Figure 2a). In  
CTL102, the CMV enhancer/promoter regulates NTR expression. CsCl-banded  
viruses were prepared and HeLa, HepG2 and SW480 cells were infected with the  
indicated mois (Figure 2b). In this case the left-to-right orientation of clone 1 was  
15 found to offer slightly greater specificity of expression, as shown, and this became  
the standard orientation adopted (CTL501). As expected CTL102 showed NTR  
expression in all three cell lines independent of their  $\beta$ -catenin status. In contrast,  
CTL501 was highly active only in HepG2 and SW480 cells. Even at a 5x higher  
moi NTR expression was barely detectable in HeLa cells. Clone 13 whilst as active  
20 as CTL501 in HepG2 and SW480 cells also expressed in Hela although still at a  
relatively low level compared to the former.

**Example 3**

*The Tcf-responsive adenovirus CTL501 shows anti-tumour activity in vivo.*

25 Having established that CTL501 can express high levels of NTR in permissive  
cells *in vitro* we tested whether intratumoral injection of HepG2 xenografts with the  
virus resulted in the expression of sufficient enzyme to sensitise the tumours to  
CB1954 and cause measurable anti-tumour effects including tumour regression. In  
the experiment 4 out of 5 tumours underwent clear regression (Figure 3). After 56  
30 days two of the four responders could be categorised as complete regressions and  
two animals harboured a quiescent, very small tumour. Tumours injected with  
vehicle only and treated with CB1954 grew out as expected. Interestingly virus

injection alone resulted in an apparent slowing of tumour growth. We attribute the variable response to the treatment to the inherent variability of the intratumoral injection technique.

#### 5 Example 4

*High level expression of NTR in CTL501- injected SW480 xenografts.*

Having demonstrated that CTL501 is highly active in SW480 colorectal cancer cells *in vitro* we determined whether high level NTR expression could be obtained by intratumoral injection of subcutaneous SW480 xenografts in nude mice. Four  
10 tumours were injected with either CTL102 or with CTL501 and 48 hours later, following humane sacrifice, were excised, fixed, sectioned and immunostained for NTR expression. The results are summarised in Figure 4. These provide further  
15 evidence that CTL501 expresses NTR at a level at least comparable to CTL102. We attribute the significant variation in the percentage of cells that are NTR positive to the inherent variability of the intratumoral injection technique.

#### Example 5

*Systemic administration of adenovirus followed by CB1954 treatment: CTL501 is much less toxic than CTL102 (CMV-NTR).*

20 We show above that intratumoral injection of CTL501 results in high level NTR expression. Associated with this method of obtaining specific delivery of a therapeutic gene to tumour cells however is the danger of virus dissemination, in particular via the bloodstream to the liver. Based on the *in vitro* specificity data presented above we predicted that whereas intravenous injection of nude mice  
25 with CTL102 would result in high level expression of NTR in the liver and consequently significant toxicity of CB1954, injection of CTL501 would be relatively very well tolerated as this should result in very little or no liver expression of NTR. The results shown in Figure 5 support this. Whereas a dose of 10E9 particles of CTL501 + CB1954 treatment resulted in virtually no toxicity, a tenfold  
30 lower dose of CTL102 resulted in 100% mortality. We conclude that CTL501 expresses no, or an insignificant amount of, NTR in normal cells, in particular the liver.

**Example 6**

*Construction of an improved Tcf-based promoter that is fully inactive in cells lacking  $\beta$ -catenin activity.*

- 5 For some applications it would be desirable to increase the specificity of the 5merTcf-based artificial promoter further, for instance to control the replication of a therapeutic adenovirus. We therefore evaluated the combination of the 5merTcf element described above with the adenoviral (Ad5) E1B gene TATA box (5merTcf-E1BTATA-Luc (CTL502), Figure 6a). As shown above, in transient transfections in
- 10 HeLa and SW480 the 5merTcf-SV40 promoter is highly active in SW480, here comparable to CMV, but shows a background activity in HeLa cells due to the basal activity of the SV40 promoter. In contrast, the 5merTcf-E1BTATA-luc construct although less active in SW480 (60% activity of 5merTcf-SV40) was completely inactive in HeLa (not deregulated  $\beta$ -catenin). Expressed another way,
- 15 replacement of the SV40 minimal promoter with the E1BTATA element resulted in an increase in inducibility in SW480 compared to HeLa from about 30-60fold for 5merTcf-SV40 to about 600-2,000fold for 5merTcf-E1BTATA i.e. a 20fold improvement.

20 **Example 7**

*The activity of the 5merTcf-SV40 and 5merTcf-E1BTATA promoter constructs is dependent on the relative positioning of the Tcf sites.*

- To determine whether changing the arrangement of the sites along the DNA helix influences the activity of 5merTcf-based promoter constructs we constructed
- 25 "TcfA", "TcfB" and "TcfC" (Figure 7) in combination with either the SV40 minimal promoter or the E1BTATA and compared their activity with the respective original 5mer Tcf-based promoters i.e. +SV40 or + E1BTATA. In transiently transfected SW480 cells, the highest level of reporter gene expression was obtained with the TcfC-E1BTATA promoter element (Figure 8a). The lowest level of expression was
- 30 obtained with the E1BTATA-TcfB combination which was about half as active as TcfC-E1BTATA. For the SV40 combinations less variation in expression was observed (Figure 8b). Furthermore in marked contrast to the result with E1BTATA,

the TcfC-SV40 promoter was the least active and the TcfB-SV40 the most active. These results suggest that the optimum spacing of the Tcf sites is dependent on the basal promoter that is combined with the Tcf elements. Further evidence for a critical role in the spacing of Tcf sites in determining the level of expression in  $\beta$ -catenin deregulated cells is provided in Figure 9. This shows the results of transient transfections of SW480 with Tcf-E1BTATA-luc constructs containing fewer than 5 Tcf sites (2, 3 and 4). Whilst there is a general increase in expression with an increasing number of sites, the results also show that by appropriate spacing of 3 sites a higher level of expression was obtained compared to an alternative arrangement of 4 sites.

None of the constructs described above exhibited an increased background expression level in Hela compared to the original 5merTcf-based promoters i.e. alteration of the spacing of the Tcf sites does not result in a loss of the exquisite specificity we have described above.

#### Example 8

*The activity of the TcfC-E1BTATA promoter element is determined by the distance between the Tcf binding sites and the E1BTATA box.*

We also determined the effect on expression of changing the distance between the Tcf binding sites and the TATA box (Figure 10). Four constructs based on the TcfC-E1BTATA were constructed and compared (Figure 10a - and see the materials and methods section). As shown in Figure 10b an inverse relationship was discovered between the Tcf-to-TATA separation and the level of luciferase expression in transfected SW480 cells. Although expression may be further improved by reducing the separation further it is likely that 25bp is approaching the minimum separation. As below this the initiation complexes and Tcf/ $\beta$ -catenin would be expected to sterically hinder access to their respective binding sites. The new constructs tested here retained the specificity of the original 5merTcf-E1BTATA artificial promoter (data not shown).

#### Example 9

*The activity and specificity of the 5merTcf-E1BTATA promoter is also retained in a replication defective adenovirus.*

To confirm that the 5merTcf-E1BTATA (CTL502) promoter also retains its activity and specificity in the context of adenovirus we infected HeLa and SW480 cells  
5 with CTL102, CTL501 and CTL502 and assayed NTR expression by ELISA (Figure 11). As expected, CTL102 was highly active in both HeLa and SW480. Consistent with transient transfection studies shown above, both CTL501 and CTL502 were highly active in SW480 but only very weakly active in HeLa. Furthermore, CTL502 was apparently less active than CTL501 in HeLa. Whereas  
10 CTL501 expressed a clearly detectable level of NTR at high-moi (1500pfu/cell) this was not detected with CTL502. The combination of  $\beta$ -catenin/Tcf responsive elements with the adenoviral (Ad5) E1BTATA box thus provides an extremely high level of tumour selectivity likely to be suitable for the expression of genes encoding highly potent therapeutic agents which could significantly damage non-cancerous  
15 cells even at low levels.

### **Example 10**

*Intravenous injection of CTL501 and CTL102 into normal mice: lack of liver expression with CTL501*

20 We show above (Example 5) that systemic administration of CTL501 to normal mice (tail vein injection) followed by CB1954 treatment is very well tolerated. In contrast, injection of a tenfold lower dose of CTL102 (CMV.NTR)/CB1954 combined with CB1954 was lethal in all cases. We concluded from this result that the  $\beta$ -catenin /Tcf-responsive promoter driving the NTR gene in CTL501 is inactive  
25 or at most weakly active in normal mouse tissues infected by the virus, principally the liver. To provide direct evidence for this, mice were injected with CTL501 or CTL102 and liver expression determined by immunostaining 48 h post-injection. In Figure 12 we show representative liver sections stained for NTR expression. Injection of CTL501 resulted in sporadic,  
30 low-level NTR expression whereas a 10 fold lower dose of CTL102 generated high level expression in a majority of cells. We interpret this result and that described in Example 5 to indicate that the  $\beta$ -catenin /Tcf-4 complex is either absent from or

present at too low a level in the nuclei of normal mouse hepatocytes to activate the promoter. It is highly unlikely that these data reflect an inability of murine beta-catenin/Tcf-4 to activate the promoter as (i) the Wnt pathway is evolutionarily highly conserved from flies upwards and (ii) the promoter of at least one murine gene that is activated by Wnt signalling (cdx) contain Tcf binding sites that fit the consensus human Tcf-4 binding site that we have used to build the promoter (Lickert *et al* (2000) *Development* 127: 3805-3813).

#### Example 11

##### 10 CTL501/CB1954 anti-tumour efficacy in a xenograft model of colorectal cancer

We show above in Example 3 that i.t. injection of HepG2 (liver cancer) xenografts with a single dose of CTL501 strongly sensitised this  $\beta$ -catenin -deregulated tumour model to the prodrug CB1954, resulting in tumour regression in the majority of cases. We show here that CTL501/CB1954 therapy is highly effective in a xenograft model of  $\beta$ -catenin-deregulated colorectal cancer (SW480). Two size randomised groups of tumours were injected with  $10^9$  and  $10^{10}$  particles of CTL501 respectively and CB1954 administered to the host mice beginning 48 h later. So as to be able to compare the efficacy observed to that achievable when NTR was expressed from the CMV promoter, two additional tumour groups were injected with equivalent doses of CTL102. As shown in Figure 13, CTL501 and CTL102 injection resulted in a similar level of anti-tumour efficacy. Non-virus injected control tumours (vehicle injected + systemic CB1954 treatment) grew strongly.

25

#### Example 12

##### CTL503/CB1954 anti-tumour efficacy in a xenograft model of colorectal cancer

We show above (Examples 6-8) that it is possible to build a  $\beta$ -catenin /Tcf-4 responsive promoter with an increased dependence on  $\beta$ -catenin /Tcf-4 (and therefore improved specificity for tumours with deregulated  $\beta$ -catenin) by substitution of the SV40 minimal promoter fragment with the Ad5 E1B TATA and

30

by altering the spacing between the Tcf binding sites and between the E1B TATA and promoter-proximal Tcf-4 binding site. A recombinant virus ("CTL503") was constructed containing this modified promoter driving NTR to determine whether the high level expression in permissive cells observed using transient transfection experiments would be retained in the context of an adenovirus backbone. In Figure 14 we show that i.t. injection of SW480 xenografts with CTL503 and subsequent CB1954 administration resulted in an anti-tumour response of a similar magnitude to that resulting from CTL102+CB1954 treatment. These *in vivo* efficacy data thus provide strong evidence that the improved tumour specificity of CTP3, detected by transient transfection experiments was gained with retention of a high level of activity in tumour cells with deregulated  $\beta$ -catenin /Tcf-4.

### Example 13

*The  $\beta$ -catenin /Tcf-dependent promoters express at very low levels in cultured primary human hepatocytes, dermal fibroblasts and endothelial cells*

We show above that the claimed promoters express at very low levels in tumour cell lines that retain normal  $\beta$ -catenin regulation. From this we infer that these promoters will be inactive/weakly expressed in normal human cells, i.e. we used the tumour cells to model normal cells. To provide direct evidence for this we determined the activity of two of the promoters in a panel of cultured primary human cells (hepatocytes, endothelial cells and dermal fibroblasts). To facilitate these and subsequent studies involving cultured primary human tissue (see Examples 15 and 16 below) we constructed recombinant adenoviruses expressing beta-galactosidase under the control of two of the claimed promoters, "Ad.CTP1-nLacZ" and "Ad.CTP3-nLacZ" (in the interest of clarity we now use a systematic nomenclature for the promoters: the original promoter present in CTL501 is renamed as "CTP1"; the optimised promoter present in CTL503 is renamed as "CTP3").

Figure 15 shows that the CMV promoter expressed strongly in all three cell types tested whereas CTP1 and CTP3 directed very low levels of beta-galactosidase expression in these cells. All three promoters were however strongly active in SW480 colon cancer cells (Figure 15 d)

**Example 14**

*CTP1 and CTP3 direct very low levels of transgene expression during growth of an E1-deleted adenovirus in the 293 and PerC6 helper cell lines*

- 5 Attempts to grow E1-deleted viruses encoding cytotoxic genes driven by promoters that are active in E1-expressing Ad helper cells are generally unsuccessful as expression of the toxic gene product prevents the cells from supporting efficient virus growth.

To determine the level of expression of the CTP1 and CTP3 promoters during  
10 virus production we infected 293 and PerC6 cells with Ad.CMV-nLacZ, Ad.CTP1-nLacZ and Ad.CTP3-nLacZ viruses and determined the level of LacZ expression 30h post-infection.

In Figure 16 we show that LacZ expression driven by CTP1 and CTP3 was significantly lower than that driven by the CMV promoter in both Ad helper lines.

- 15 However, as observed in all other non-permissive cell lines, CTP3 was clearly less active than CTP1 (approximately 10 fold). Both promoters were approximately 3 fold more active in PerC6 than in 293 cells. These data suggest that both cell lines but in particular 293 cells would support the efficient replication of first generation Adenovirus vectors encoding a toxic transgene under the control of the CTP3  
20 promoter.

**Example 15**

*CTP1 and CTP3 promoters are highly active in freshly excised metastatic colorectal cancer tissue but inactive in associated liver tissue*

- 25 Established tumour cell lines are useful model systems to study gene expression patterns in cancer. These lines are however cloned from primary cancers, which are polyclonal populations of genetically diverse and genetically unstable cells. They have also generally been continuously cultured for long periods of time, providing further scope for the selection of cells that are well adapted to ex vivo  
30 culture but not very representative of the cancer from which they were derived. For these reasons we determined the activity of CTP1 and CTP3 in freshly explanted samples of primary and secondary colorectal cancer. Samples were prepared and



infected with Ad.CMV-nLacZ, Ad.CTP1-nLacZ and Ad.CTP3-nLacZ viruses and analysed for nuclear beta-galactosidase expression as described in the Materials and methods section. Ad.CMV-nLacZ was used to determine the viability of each sample and susceptibility to adenovirus infection and to allow a comparison of the relative activities of CMV and CTP1/3 promoters. Figure 17 shows the results obtained with a secondary cancer isolated from the liver. For each virus treatment, tumour with attached liver margin was incubated in the virus suspension. As observed with all other tumour specimens, the tissue was free of endogenous beta-galactosidase activity (mock-infected samples not stained by X-Gal).

Incubation with Ad.CMV-nLacZ resulted in strong staining of both tumour and attached liver. A striking demonstration of tumour specificity was provided by infection of equivalent samples with Ad.CTP1-nLacZ and Ad.CTP3-nLacZ viruses: in both cases exposure of liver and tumour tissue resulted in a level of expression equivalent to CMV but restricted to tumour tissue. To date, all 5 colorectal metastases examined were permissive for high-level CTP1 and CTP3 expression. Of 10 primaries, 3 were found to be weakly or non-permissive for promoter activity. Of note, the primary tumours that gave rise to the secondaries were permissive in each case.

## Example 16

*High-level CTP-mediated expression correlates with high level, non-membranous expression of beta-catenin*

In Example 15 we demonstrate that CTP1 and CTP3 can provide high-level gene expression selectively in secondary colorectal cancer tissue despite the

simultaneous introduction of the transgene into neighbouring healthy liver tissue. Whilst to date the promoters were active in all secondary CRC deposits, low-level or undetectable expression was observed in 3 of 10 primaries. Analysis of these non-permissive tumours for beta-catenin revealed a correlation between the overall level and sub-cellular distribution of the protein. Figure 18 shows the

results for representative permissive and non-permissive tumour samples. Tumour A (non-permissive) is relatively well-differentiated with beta-catenin staining restricted largely to the cell periphery, consistent with this being associated with E-

cadherin. Tumour B (permissive) in contrast is poorly differentiated with a significantly higher level of cytoplasmic/nuclear beta-catenin staining. This finding provides further evidence for a dependence of the CTP promoters on beta-catenin deregulation. It conflicts with the simple model of colon carcinogenesis in which

5 beta-catenin deregulation resulting in constitutive activation of genes responsive to beta-catenin/Tcf is the initiating event. A practical application of this finding is that it may provide the basis for pre-selection of patients possessing tumours that are permissive for the CTP promoters and thus potentially treatable by a gene therapy approach in which a therapeutic gene is under the control of a CTP promoter.

## CLAIMS

1. A nucleic acid construct comprising: a TCF response element comprising:  
at least one TCF binding element having the sequence CTTTGNN,  
5 wherein N is A or T;  
and a promoter, and  
an expressible therapeutic gene operably linked to the TCF response  
element,  
wherein the TCF response element enables inducible expression of the  
10 operably linked therapeutic gene.
2. The nucleic acid construct of claim 1 wherein the therapeutic gene encodes a  
toxin or a prodrug activating enzyme.
- 15 3. The nucleic acid construct of claim 1 or claim 2, wherein the therapeutic gene  
encodes a nitroreductase capable of activating CB1954.
4. The nucleic acid construct of any one of the previous claims wherein the  
promoter is selected from the group consisting of the SV40 promoter, the E1B  
20 promoter, and the *c-Fos* promoter.
5. The nucleic acid construct of claim 4, wherein the promoter is the E1B  
promoter.
- 25 6. The nucleic acid construct of any one of the previous claims wherein the TCF  
response element comprises between 5 and 15 TCF binding elements
7. The nucleic acid construct of any one of the previous claims wherein the TCF  
response element comprises between 5 and 10 TCF binding elements.  
30
8. The nucleic acid construct of any one of the previous claims wherein the TCF  
response element comprises 5 TCF binding elements.

9. The nucleic acid construct of any one of the previous claims wherein the TCF binding elements are separated from each other by between 3 and 20 nucleotides.
- 5
10. The nucleic acid construct of any one of the previous claims wherein the TCF binding elements are separated from each other by between 3 and 12 nucleotides.
- 10 11. The nucleic acid construct of any one of the previous claims wherein the TCF binding elements are separated from each other by between 10 and 12 nucleotides.
12. The nucleic acid construct of any one of the previous claims wherein the TCF binding element closest to the promoter is between 140 and 10 nucleotides from the TATA box of the promoter.
- 15
13. The nucleic acid construct of any one of the previous claims wherein the TCF binding element closest to the promoter is between 100 and 10 nucleotides from the TATA box of the promoter.
- 20
14. The nucleic acid construct of any one of the previous claims wherein the TCF binding element closest to the promoter is between 50 and 10 nucleotides from the TATA box of the promoter.
- 25
15. The nucleic acid construct of any one of the previous claims wherein the TCF binding element closest to the promoter is between 30 and 15 nucleotides from the TATA box of the promoter.
- 30 16. The nucleic acid construct of any one of claims 1-10 or 12-15 wherein the TCF binding elements are separated from each other by between 3 or 4

nucleotides and wherein the TCF binding element closest to the promoter is 25 nucleotides from the TATA box of the promoter.

17. The nucleic acid construct of any one of the previous claims wherein the TCF  
5 binding element has the nucleotide sequence CTTTGAT.

18. A TCF response element comprising:  
at least five TCF binding elements; and  
a promoter sequence,

10 wherein the TCF response element when operably linked to an expressible gene gives inducible expression of the operably linked gene.

19. A TCF reporter construct comprising the TCF response element of claim 17  
operably linked to a reporter gene.

15

20. Use of the TCF reporter construct of claim 18 in a method of identifying candidate drugs for use in the treatment of cancers associated with the deregulation of the Wnt signalling pathway comprising the steps of :

20 contacting the TCF reporter construct with a test compound under conditions in which the reporter gene is transcribed; and measuring the transcription of the reporter gene;

wherein a test compound which inhibits transcription of the reporter gene is a candidate drug for cancer treatment.

25 21. A vector comprising the nucleic acid construct of any one of claims 1 to 16 or the TCF responsive element of claim 17 operably linked to an expressible gene.

22. A host cell transfected with the vector of claim 20.

30

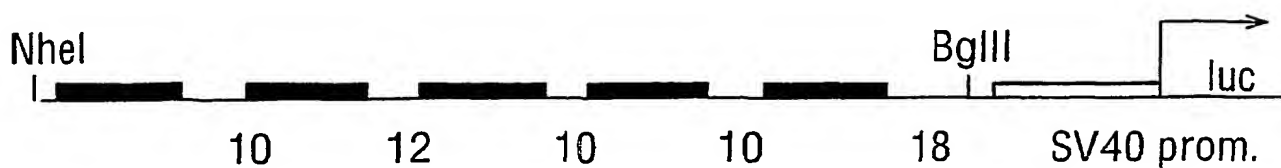
23. The nucleic acid construct of any one of claims 1 to 16, the vector of claim 20 or host cell of claim 21 for use in therapy.

24. Use of the nucleic acid construct of any one of claims 1 to 16, the vector of claim 20 or host cell of claim 21 in the manufacture of a composition for use in the treatment of cancer.
- 5
25. A method of treatment, comprising administering to a patient in need of such treatment an effective dose of the nucleic acid construct of any one of claims 1 to 16, the vector of claim 20 or host cell of claim 21.
- 10 26. A pharmaceutical composition comprising the nucleic acid construct of any one of claims 1 to 16, the vector of claim 20 or host cell of claim 21 in combination with a pharmaceutically acceptable recipient.
- 15 27. A composition for delivering the nucleic acid construct of any one of claims 1 to 16 or the TCF response element of claim 18 to a cell.

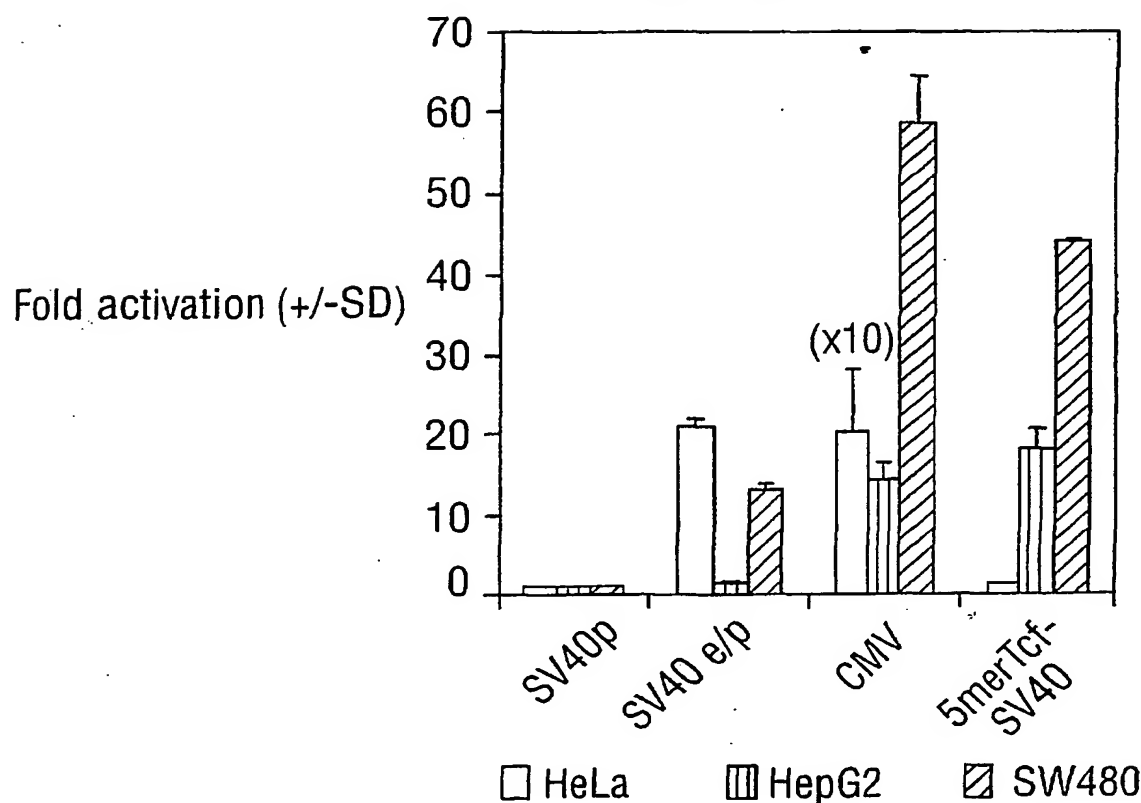
1/23

## FIG. 1a

5merTcf-SV40-Luc reporter construct (CTL501):



## FIG. 1b



## FIG. 1c

**5merTcf-SV40 (CTL501) antisense strand**

TTGAGATGCAGATCGCAGATCTGATAAAGGTGCATTAGAGATCAAAG  
 GTAGGACTCTTGATCAAAGGACTGAATTCCTTGATCAAAGGTGCATTAG  
 AGATCAAAGGTAGGACTCTTTGATCAAAGGGACTAGTAAGCTTGCTAGC  
 ACGCGTAAGAGCTCGGTACC

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FIG. 2a

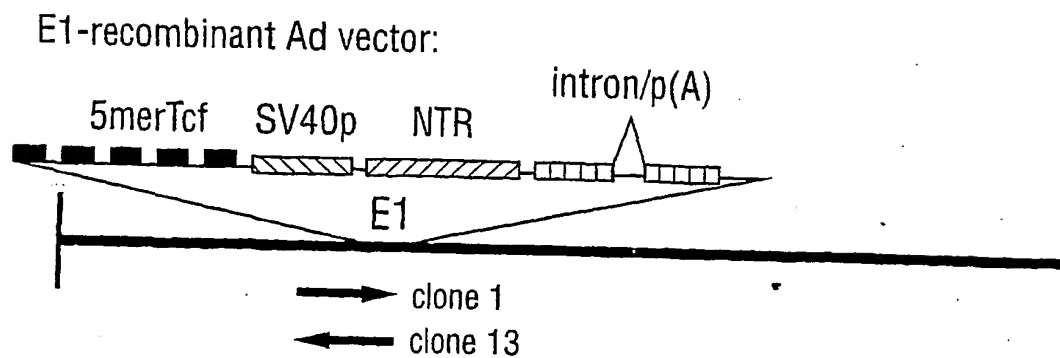


FIG. 2b

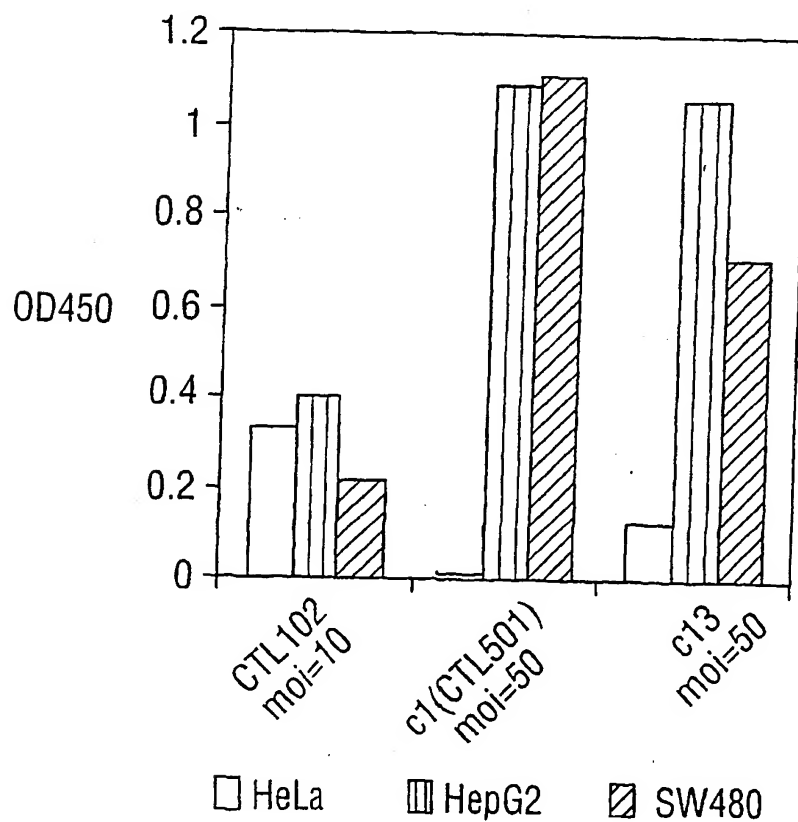




FIG. 3b

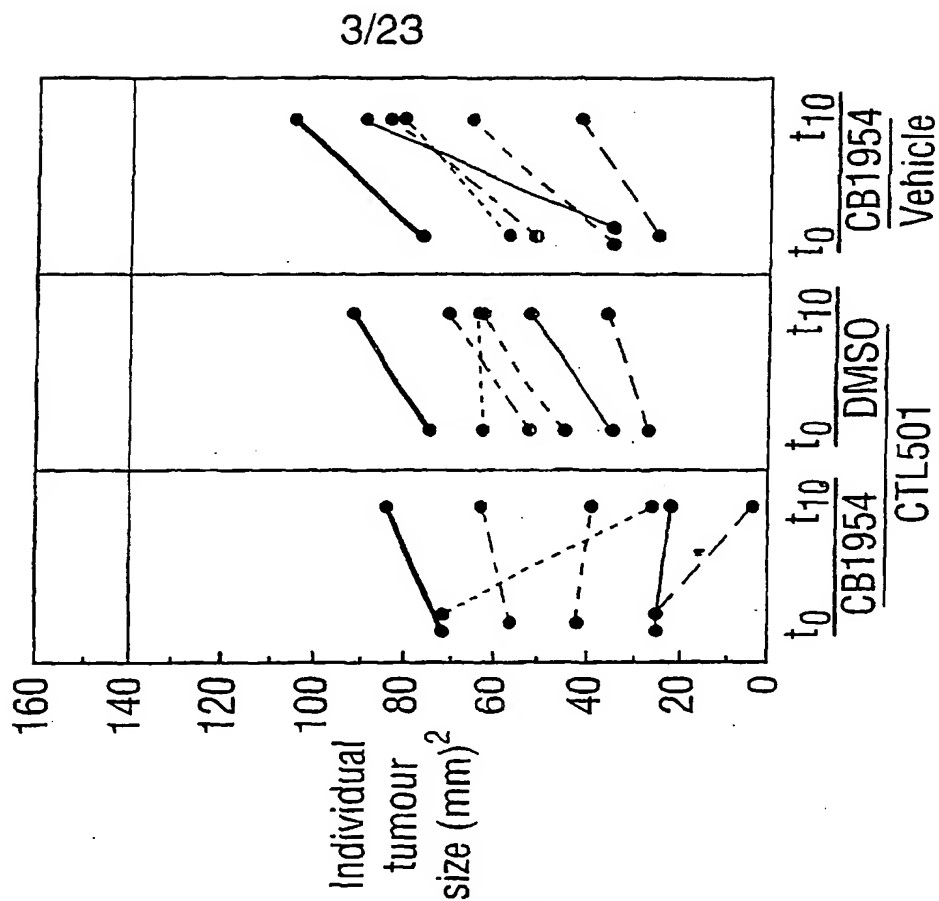
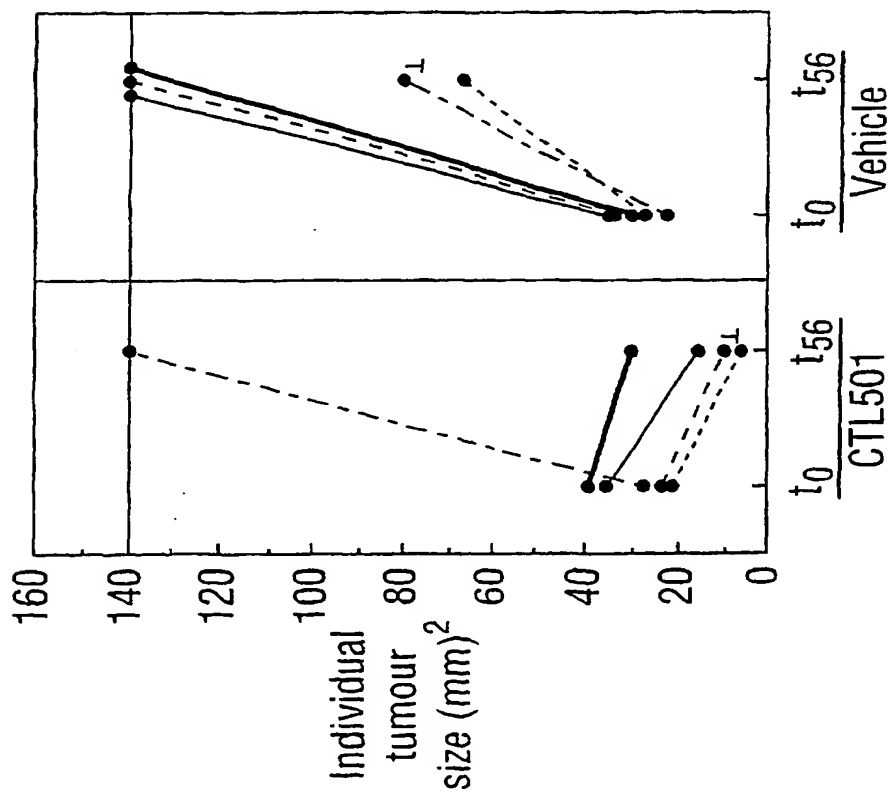
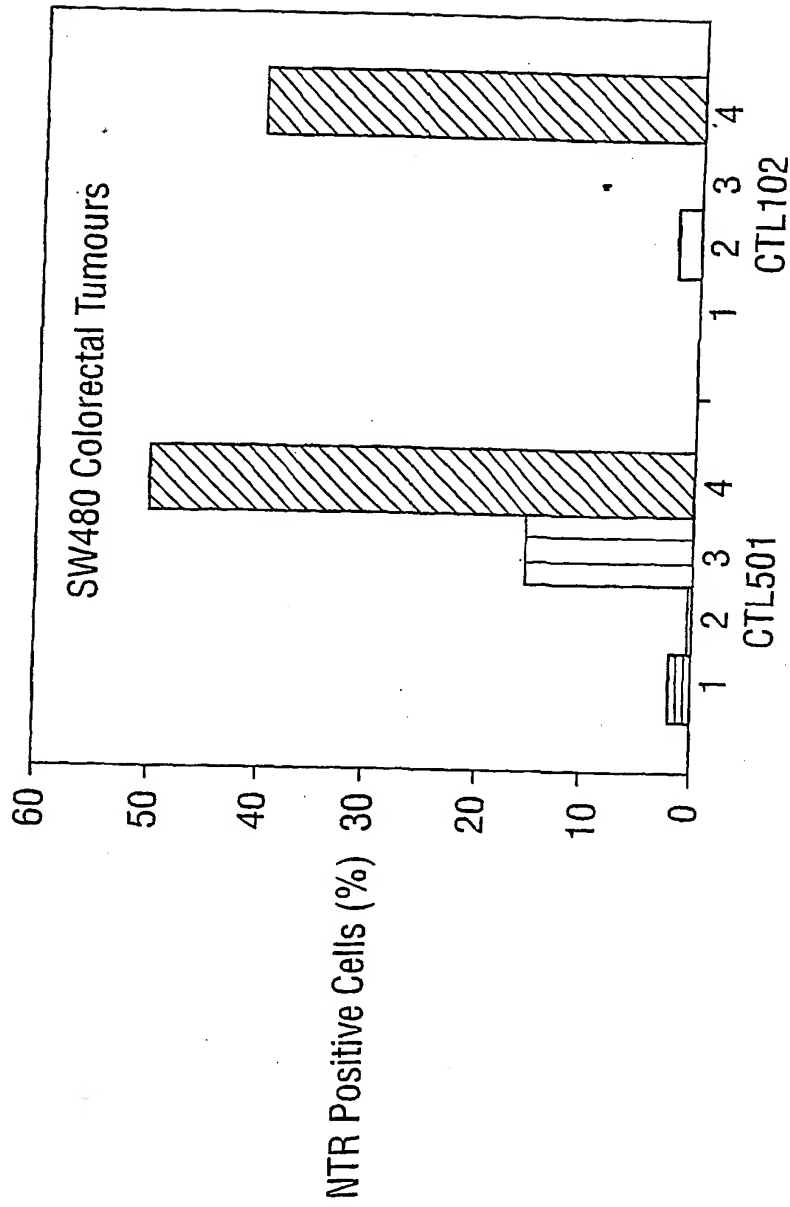


FIG. 3a



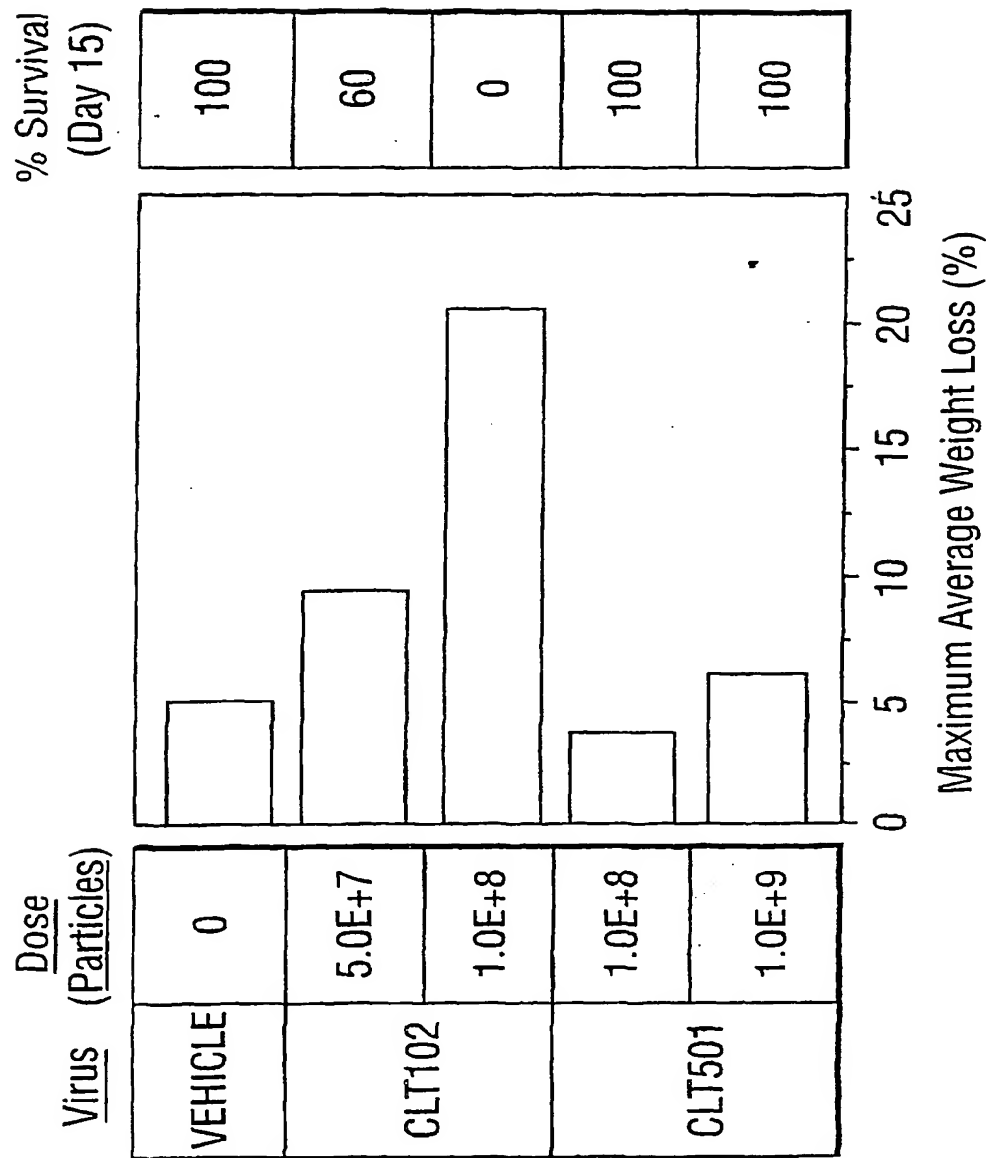
4/23

FIG. 4



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FIG. 5



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FIG. 6a

5merTcf-E1BTATA-Luc reporter construct (CTL502):

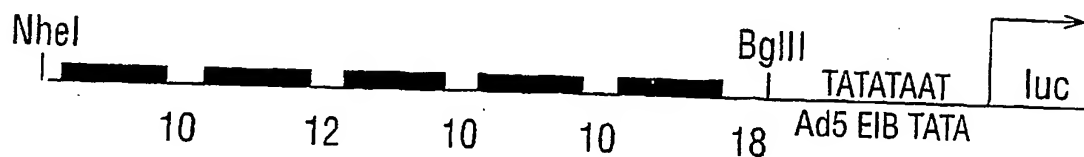


FIG. 6b

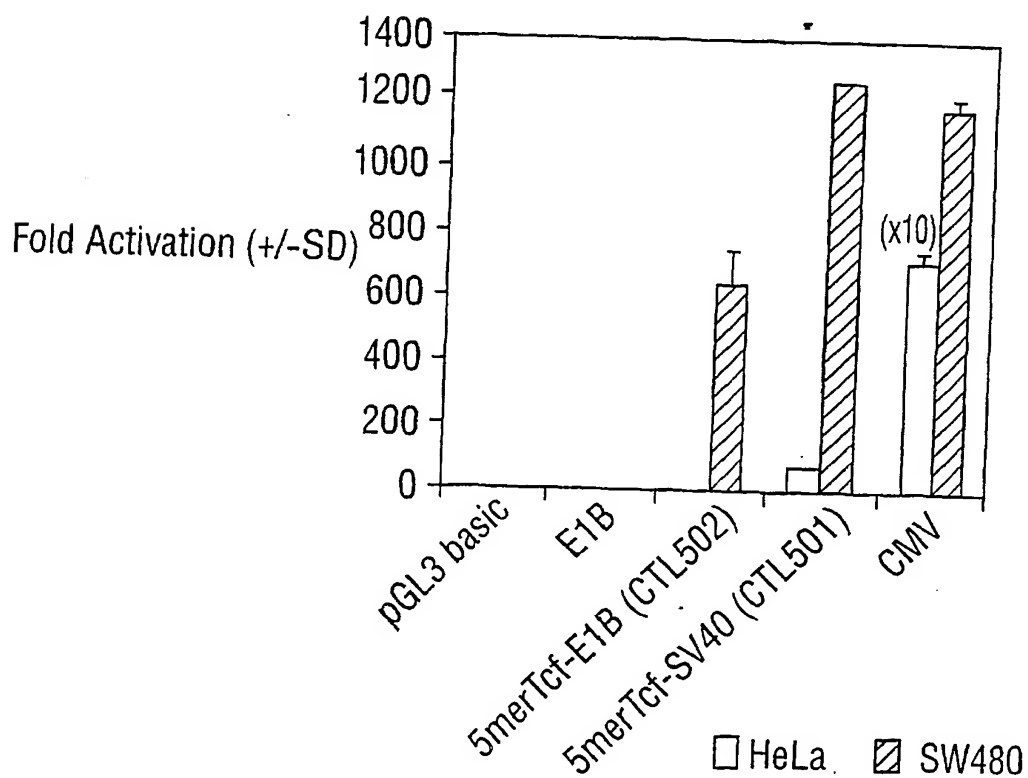
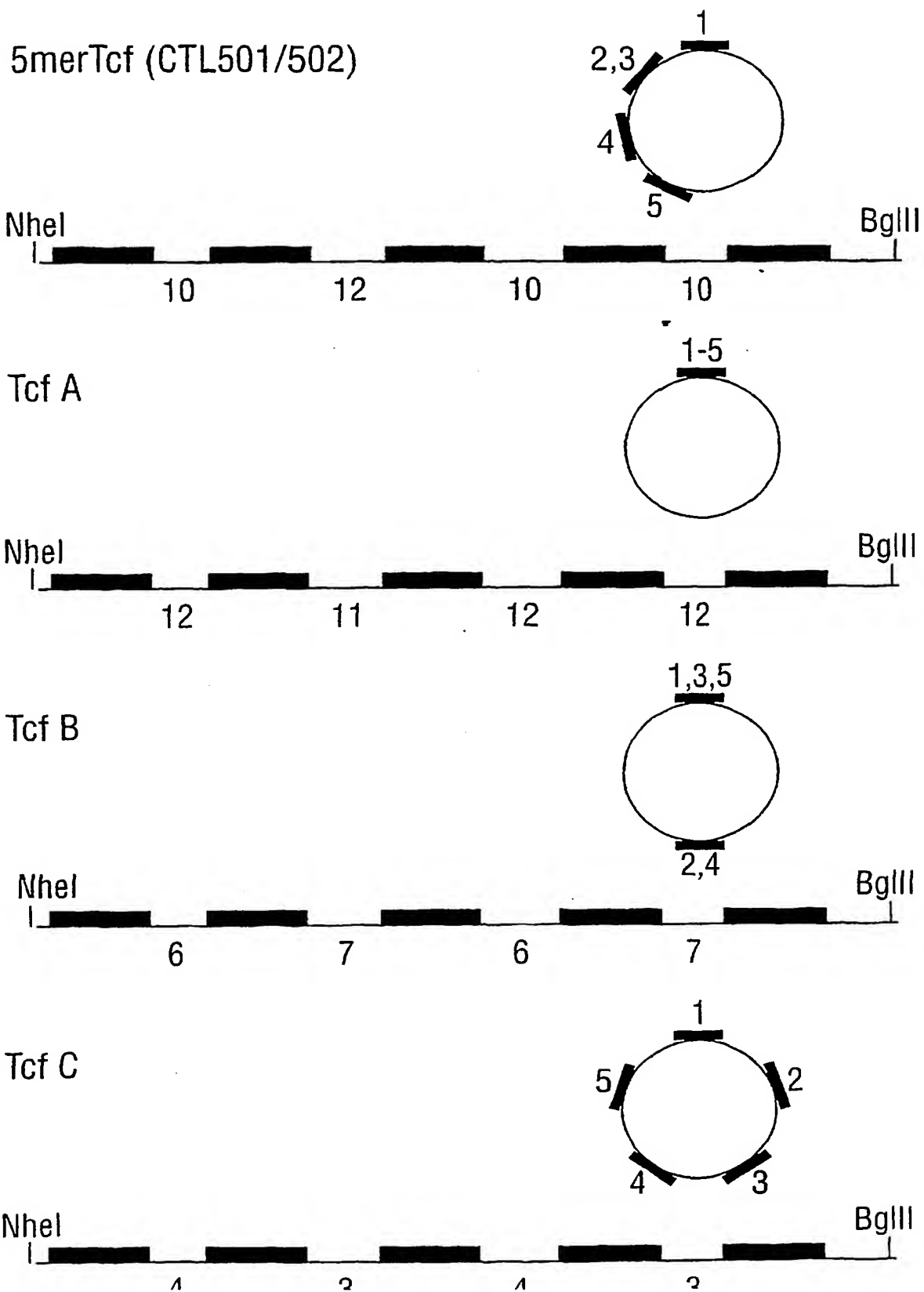


FIG. 6c

**E1BTATA clone 2 antisense strand**

GGATGCCAAGCTTTTGTAGCTTCCTTAGCTCCTGAAAATCTCGCCAAGCTGA  
 TGAATTCGAGCTGGCGCATTATATACCTCTAGAGTCGACGGATCGAGATC  
 TCGAGCCCGGGCTAGCACGCGTAAGAGCTCGGTACC

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FIG. 7

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FIG. 8a

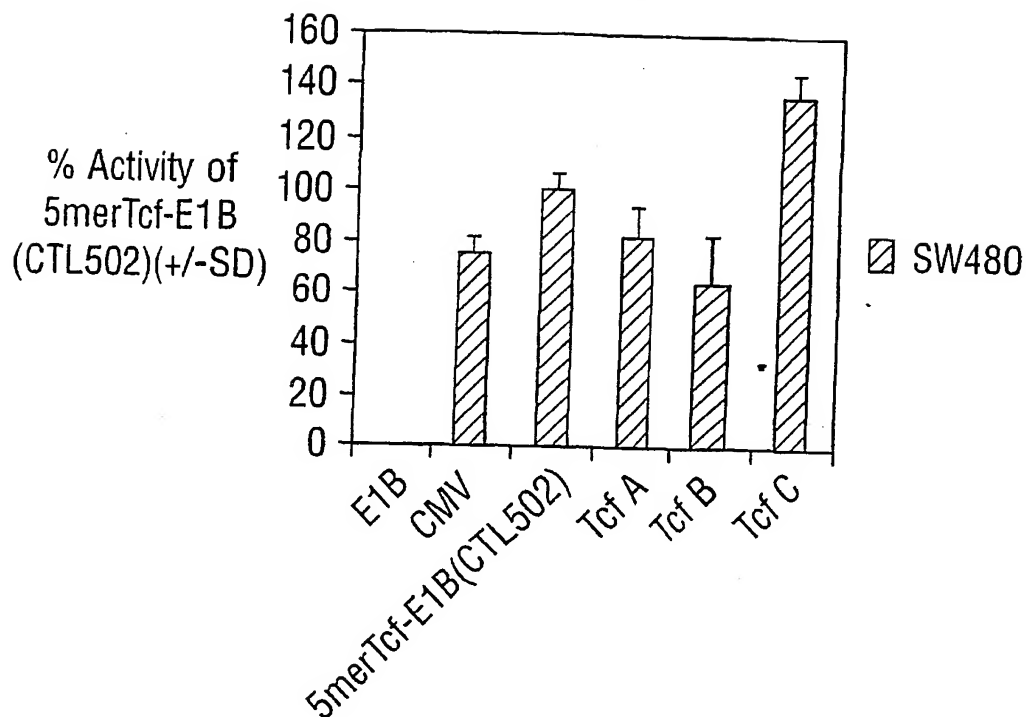
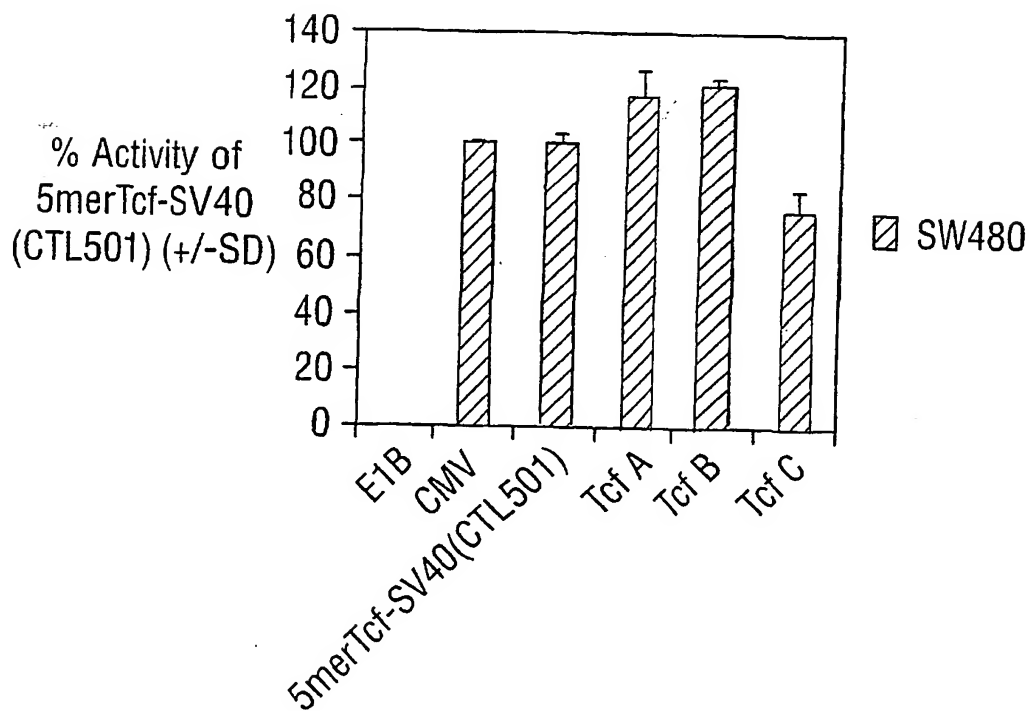
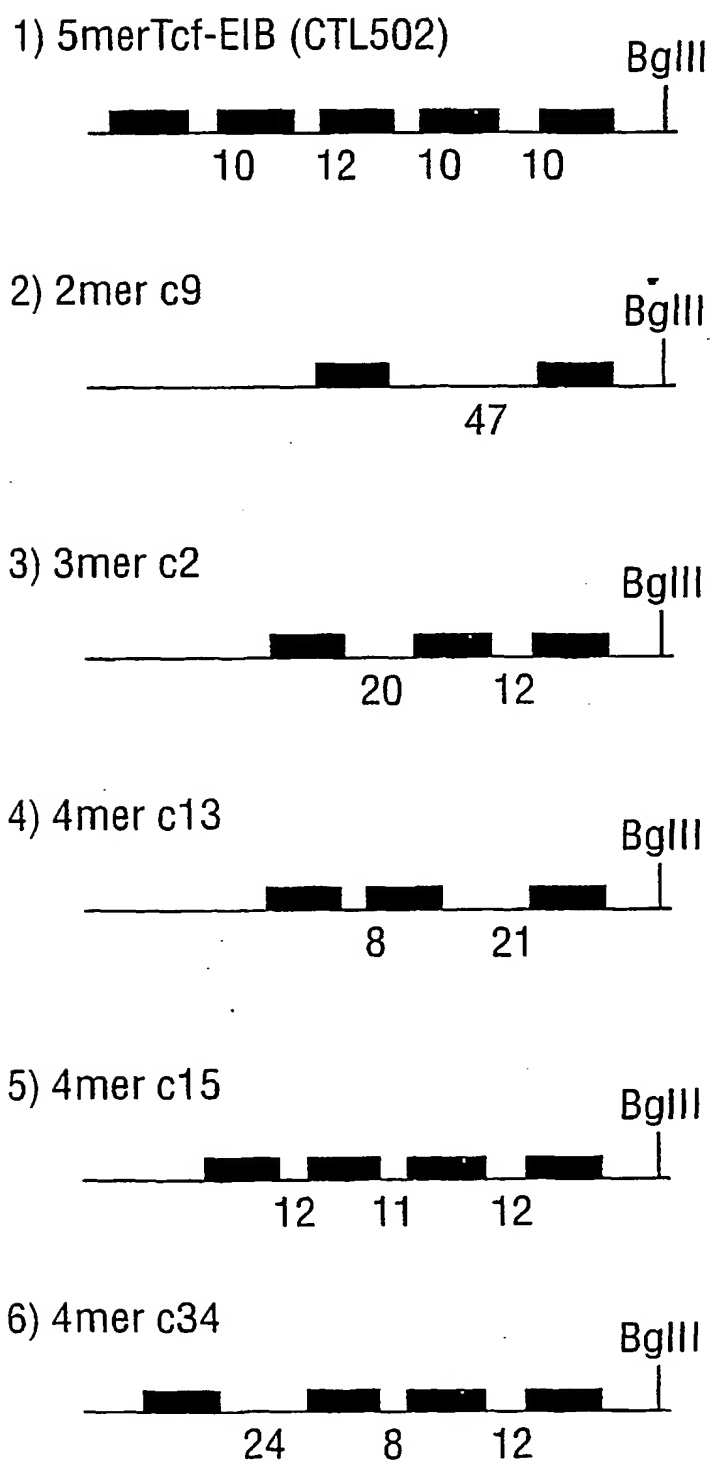


FIG. 8b



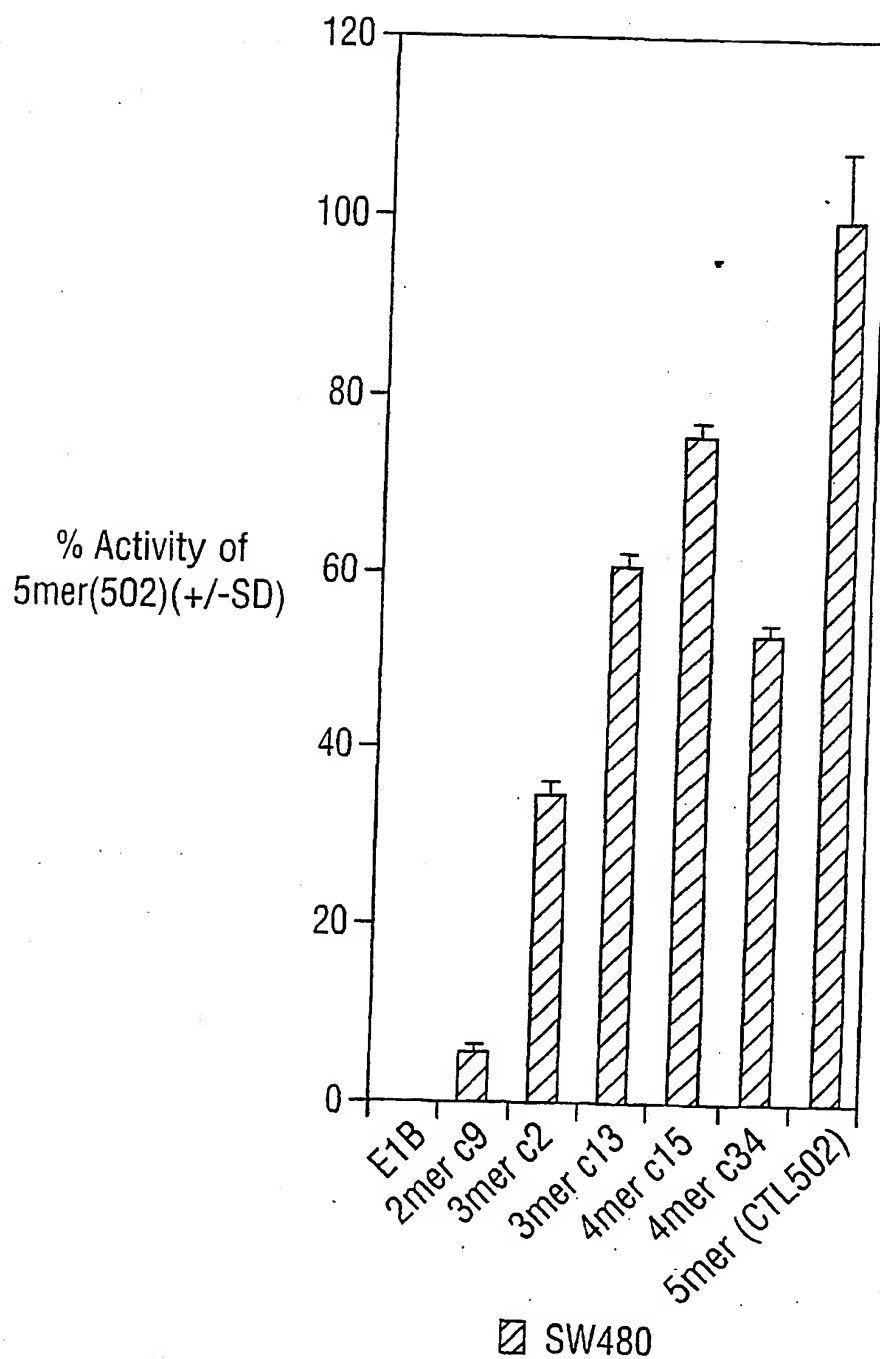
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FIG. 9a



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FIG.9b





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## FIG. 9c (i)

**2merTcf-E1BTATA clone 9 antisense strand**

ATCGAGATCTGATAAAGGTGCATTAGAGGATCAAAGGTAGGTTACTCTTTG  
AATTCAGGTGCAATTTAAAGGTAGGAACTCTTGATCAAAGGACTAGTAAG  
CTTGCTAGCACGCGTAAGAGCTCGGTACC

**3merTcf-E1BTATA clone 2 antisense strand**

ATCGAGATCTGATAAAGGTGCATTAGAGGATCAAAGGTAGGTTACT  
CTTGATCAAAGGACTGAATTCAGGAACTCTTGATCAAAGGACTAGTAAGCT  
TGCTAGCACGCGTAAGAGCTCGGTACC

**3merTcf-E1BTATA clone 13 antisense strand**

ATCGAGATCTGATAAAGGTGCATTAGAGGATCAAAGGTAGTCACAGGTGC  
AATTTAGAGGATCAAAGGTAGGAATTGATCAAAGGATAGTAAGCTTGCTAGC  
ACGCGTAAGAGCTCGGTACC

**4merTcf-E1BTATA clone 15 antisense strand**

ATCGAGATCTGATAAAGGTTCTTGATCAAAGGACTGAATTCCTTGATCAA  
GGTGCAATTTAGAGATCAAAGGTAGGAACTCTTGATCAAAGGACTAGTAA  
GCTTGCTAGCACGCGTAAGAGCTCGGTACC

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FIG. 9c (ii)

**4merTcf-E1BTATA clone 34 antisense strand**

ATCGAGATCGATAAAGGTGCATTTAGACGATCAAAGGTAGGTTACTCTTGA  
TCAAAGGAATTCCTTGATCAAAGGTGCAATTTAGAGAAGGTAGGAACTCT  
TGATCAAAGGACTAGTAAGCTTGCTAGCACGCGTAAGAGCTCGGTACC

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FIG.10a

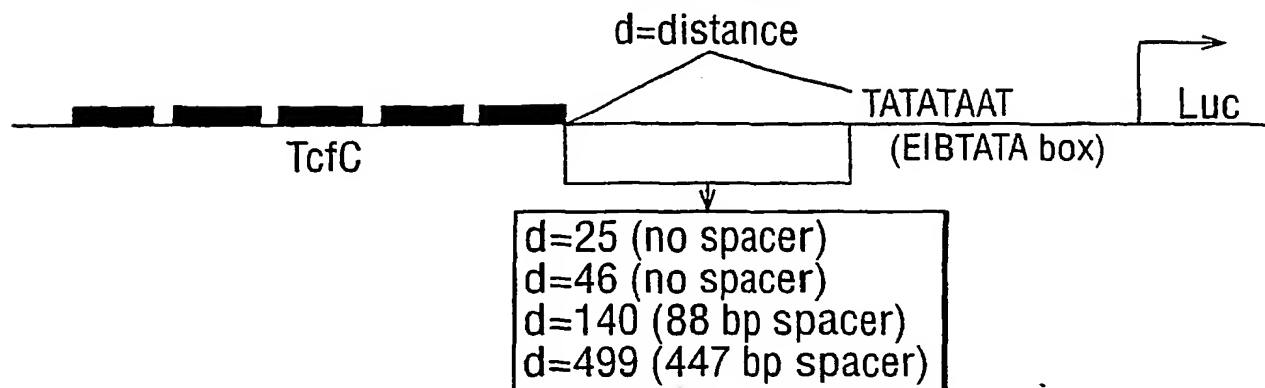


FIG.10b

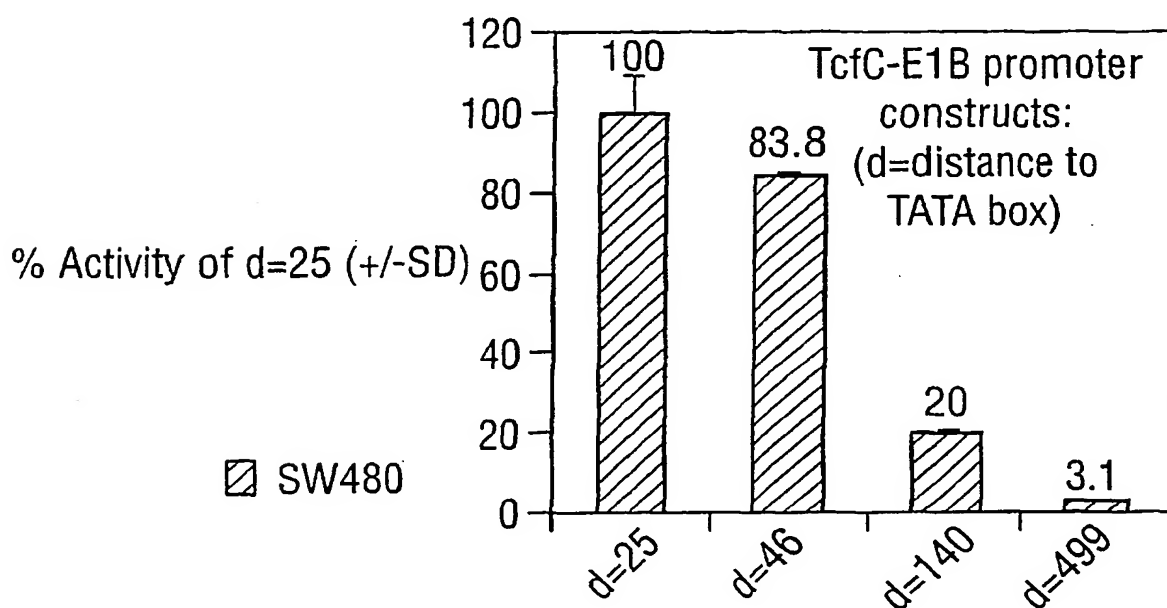


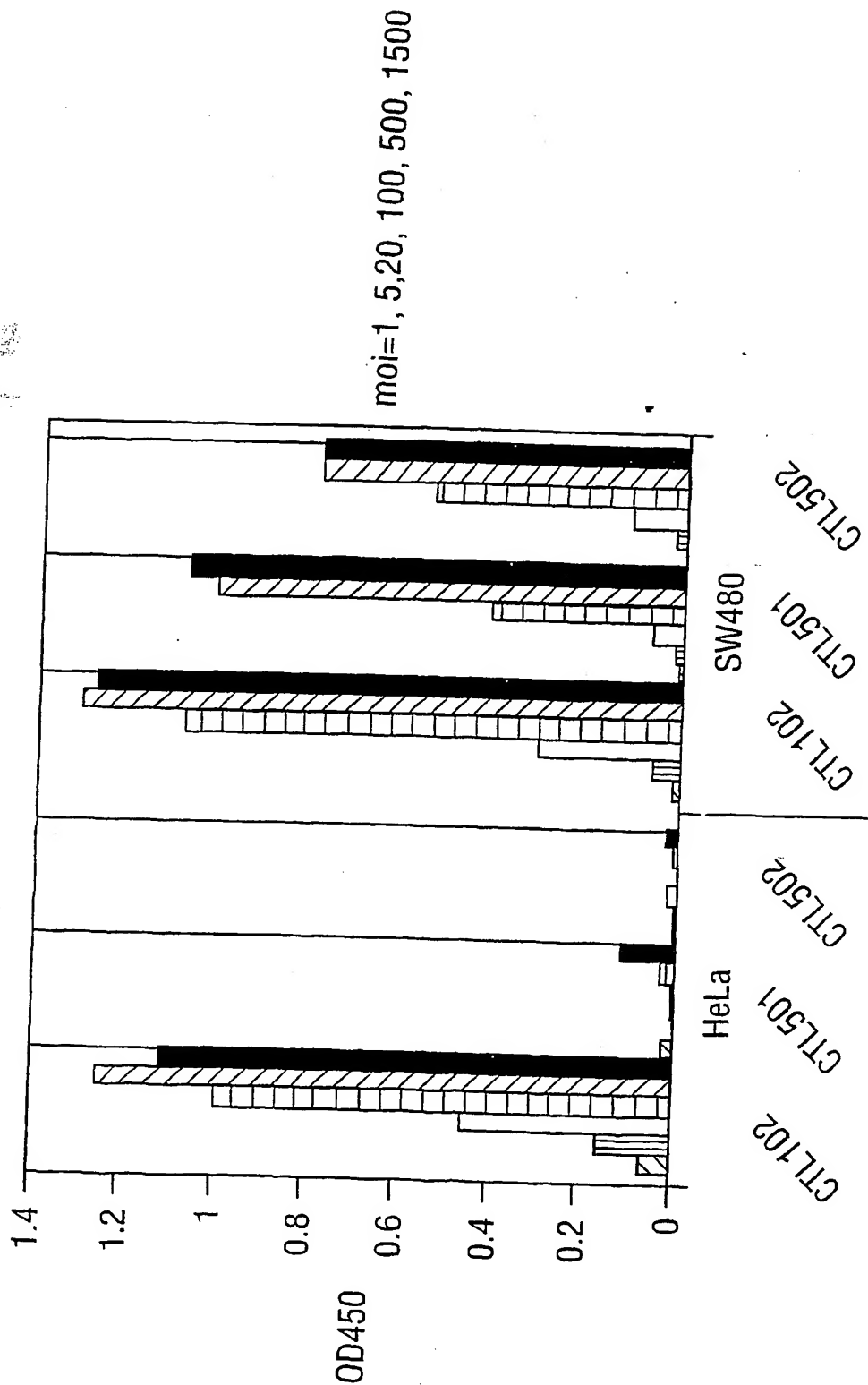
FIG.10c

**/TcfC-E1BTATA (d=25) clone 6 antisense strand**

TACCAACAGTACCGGAATGCCAAGCTAGCTTTTTAGCTTCCTTAGCTCCTG  
 AAAATCTCGCCAAGCTGATGAATTCGAGCTGGCGCATTATATACCCTCTG  
 ATAAAGGTGCATTTAGAGATCAAAGGTATGATCAAAGGACTTGATCAAAGGT  
 GAGATCAAAGGTATTGATCAAAGGACTAGAGCTTACTTAGATCGCAGATCTC  
 GAGCCCGGGCTAGCACGCGTAAGAGCTCGGTACCTATCG

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FIG.11



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FIG.12a

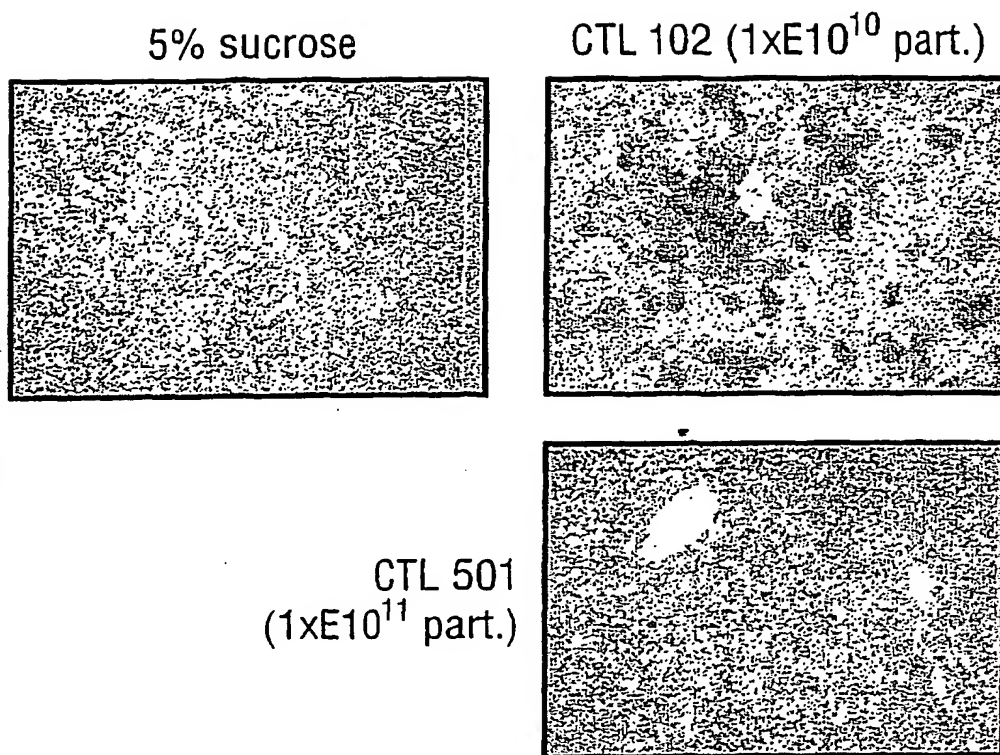
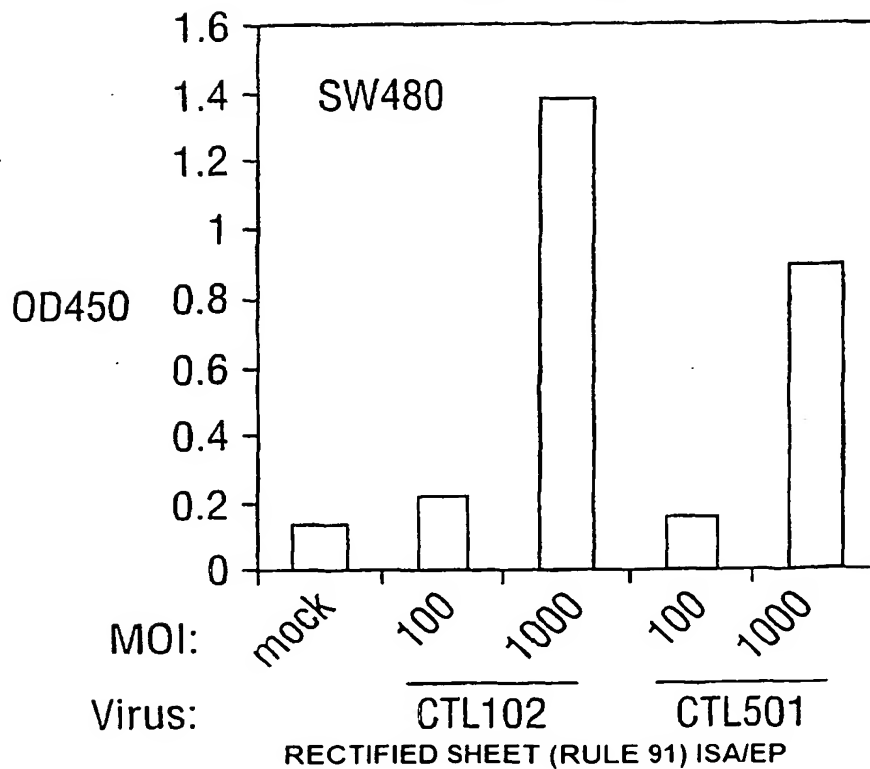
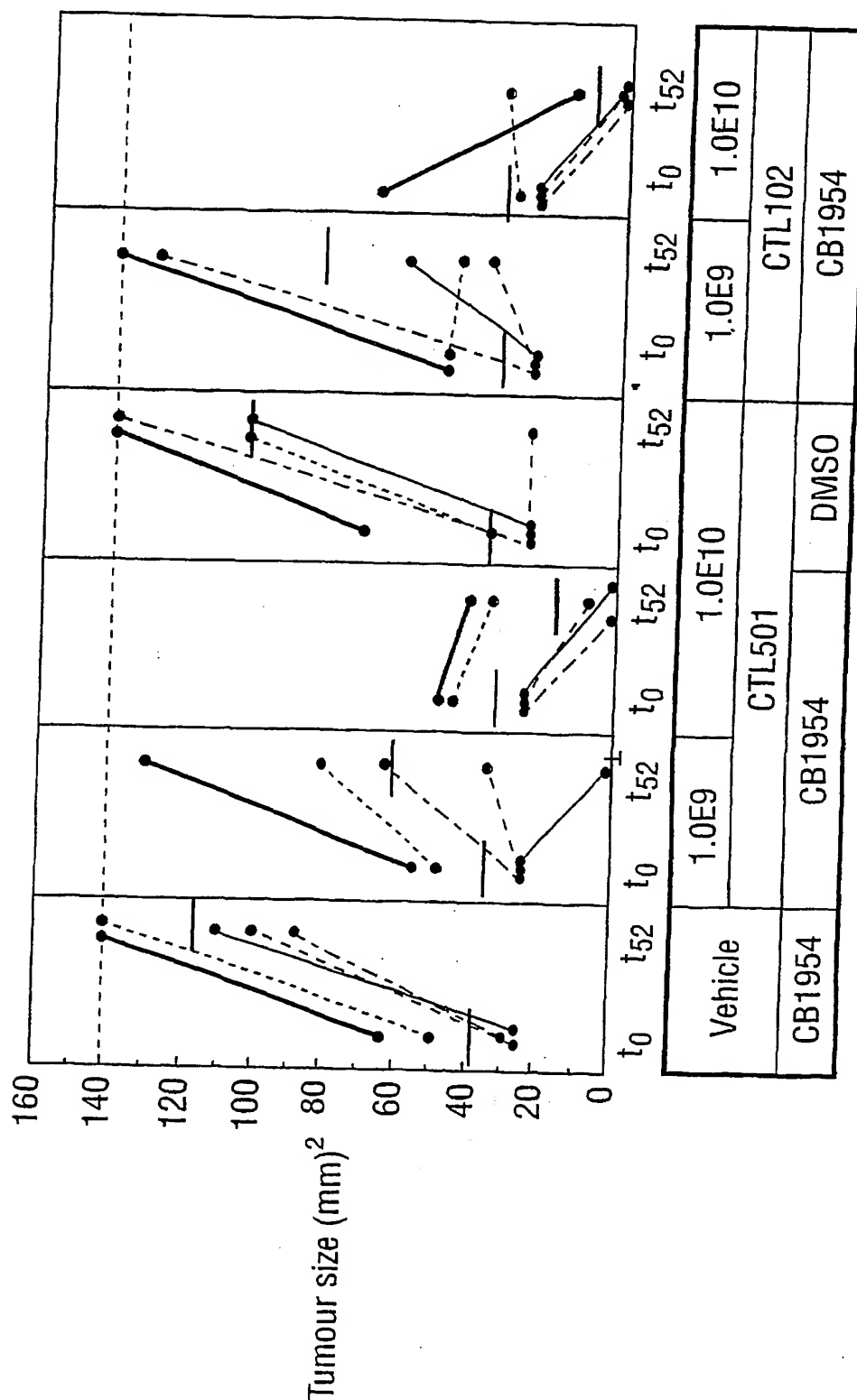


FIG. 12b



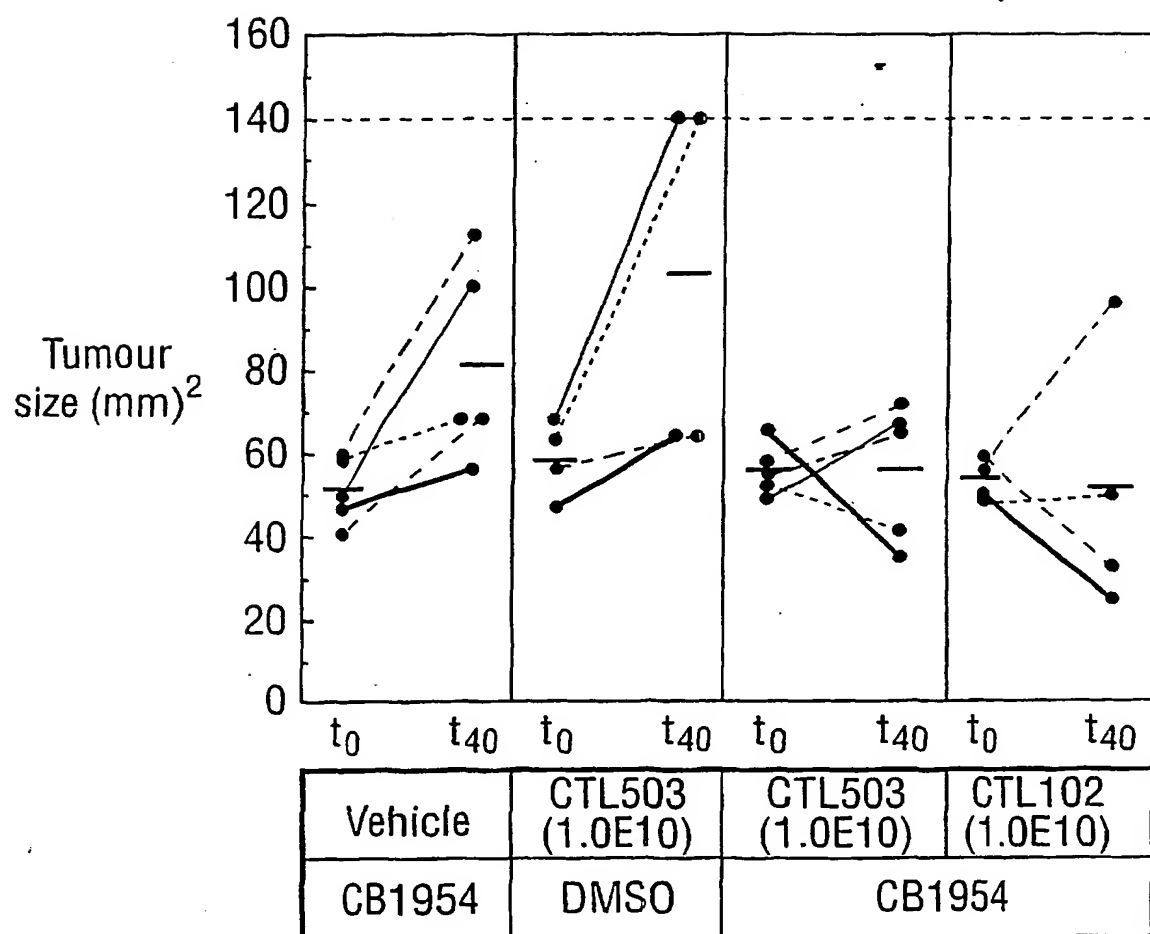
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FIG. 13



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FIG. 14



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FIG. 15a

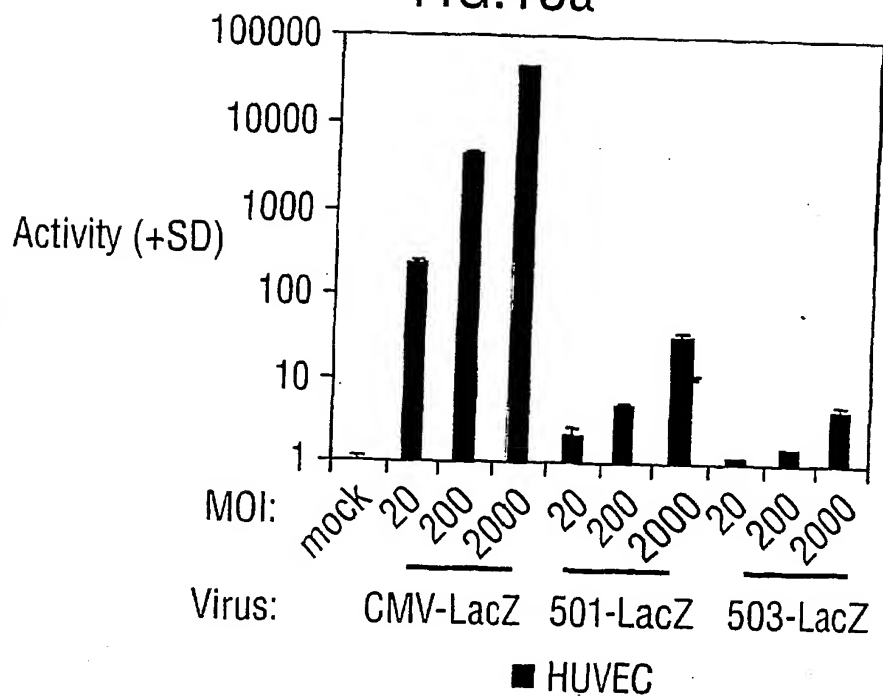
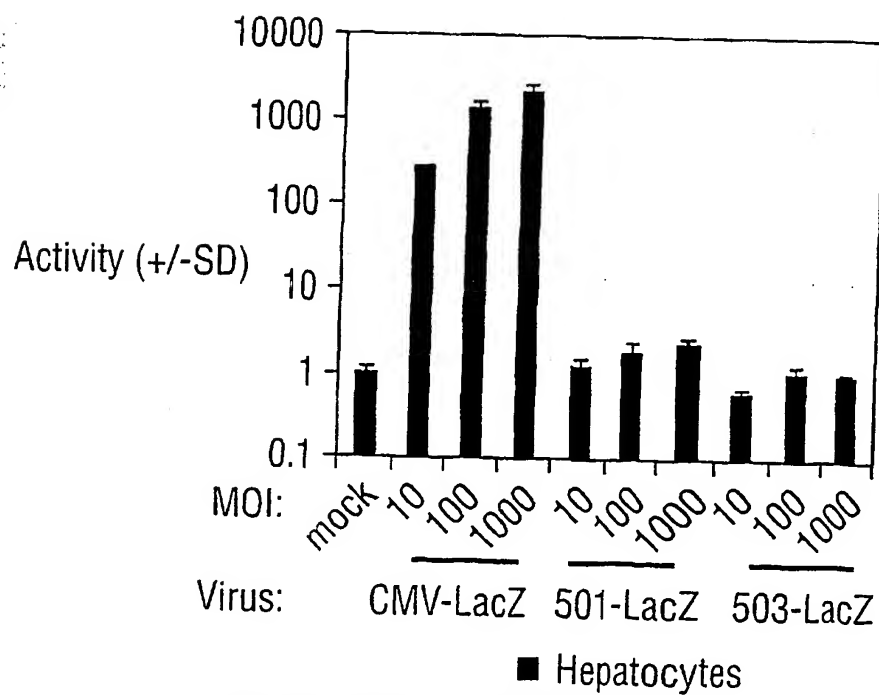


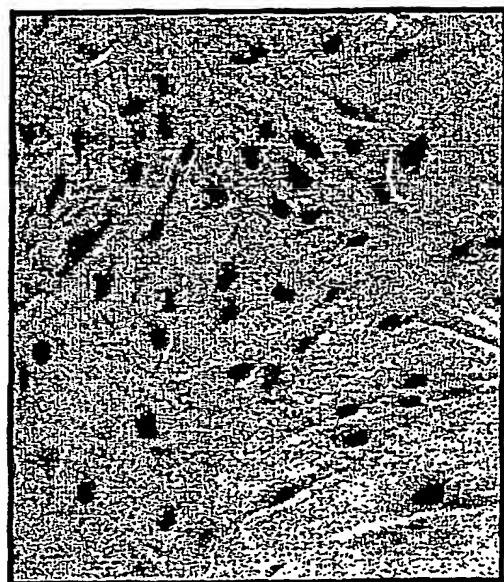
FIG. 15b



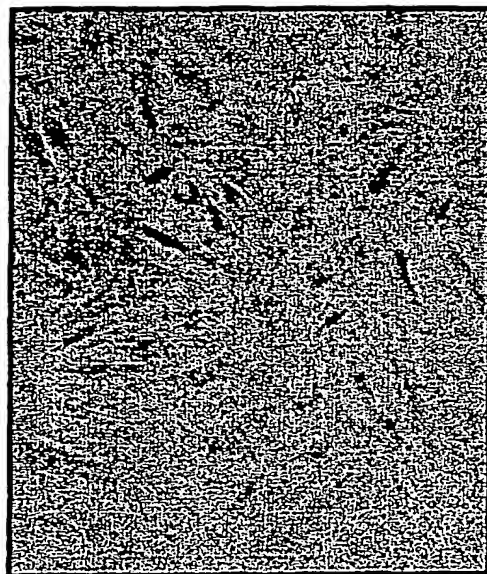


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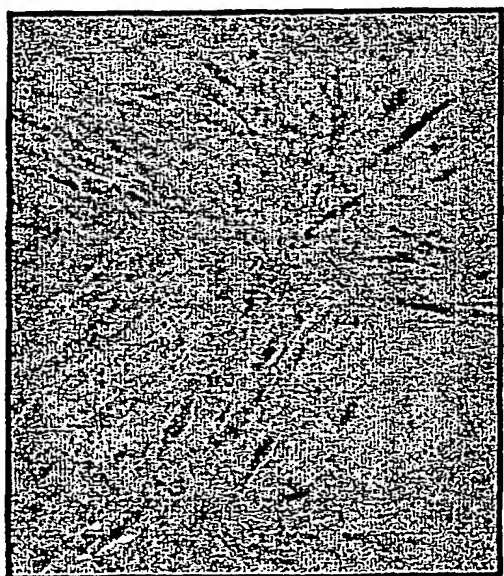
FIG. 15C - Primary human dermal fibroblasts



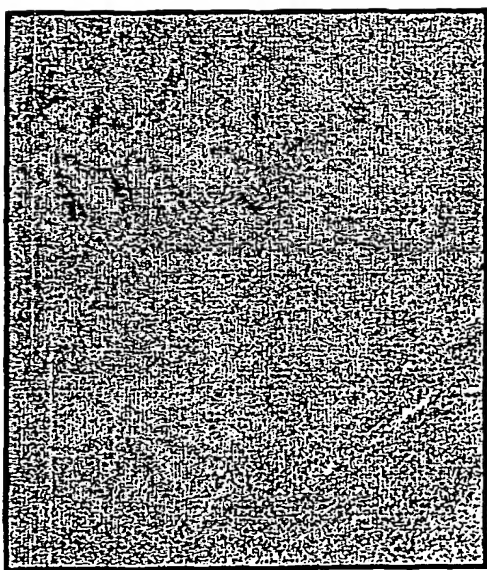
Ad-CMV-nLacZ



Ad.CTP3-nLacZ



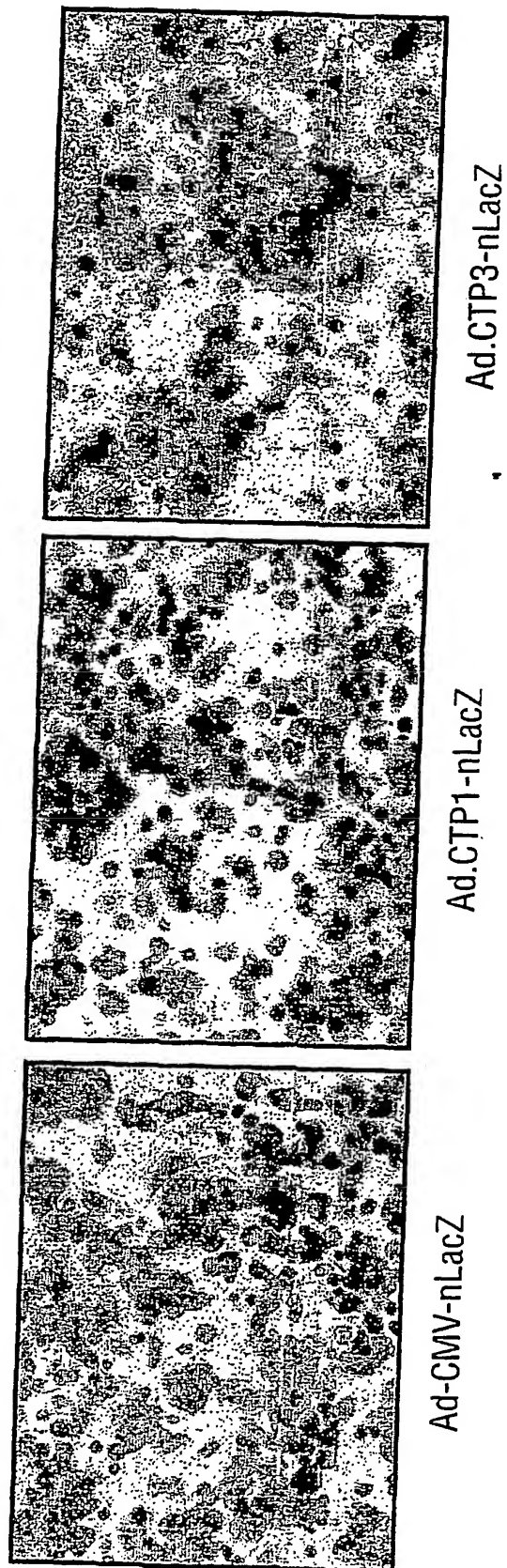
Control



Ad.CTP1-nLacZ

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FIG. 15d - SW480 colorectal cancer positive control



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FIG.16a

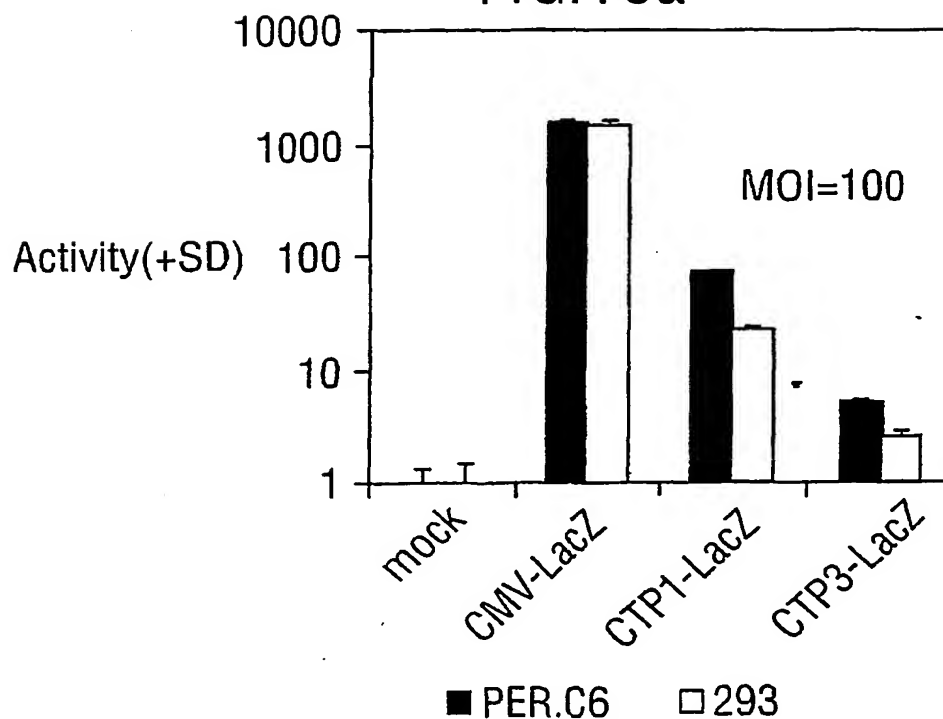
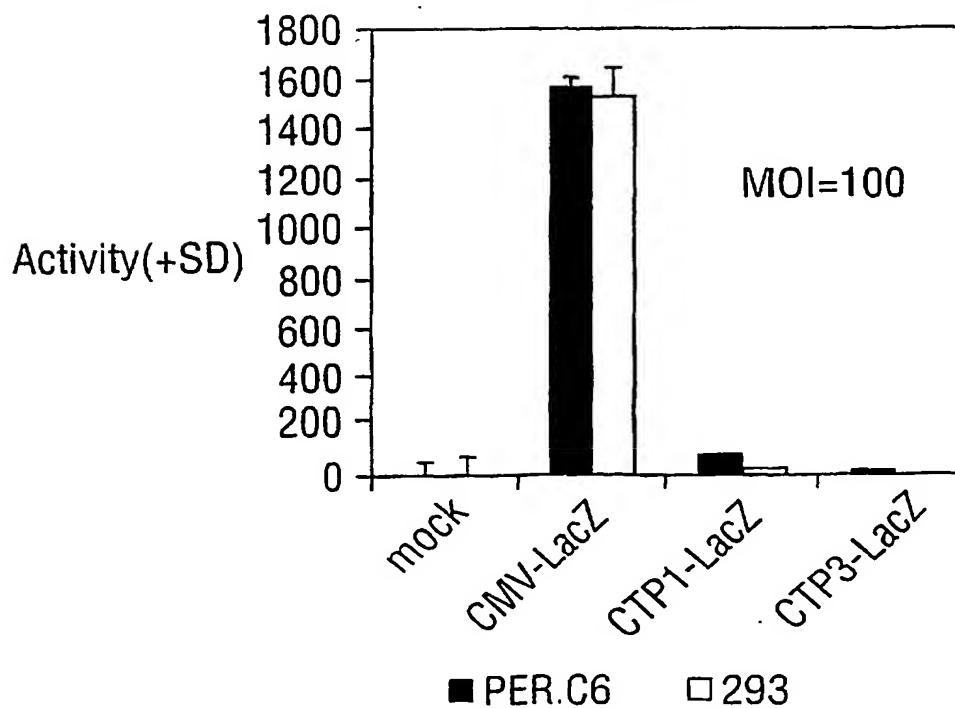
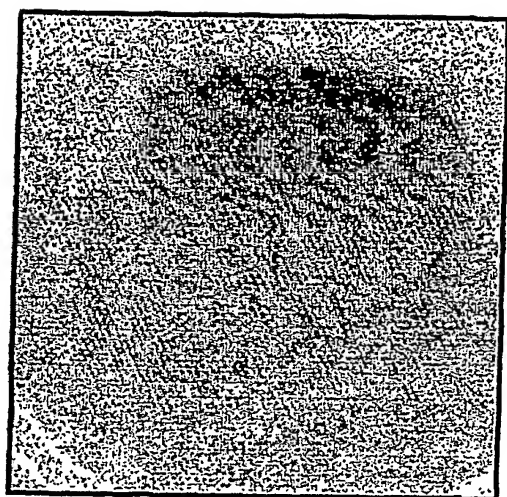


FIG. 16b

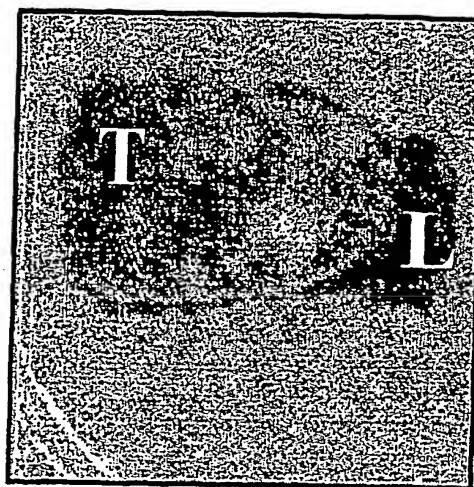


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FIG.17

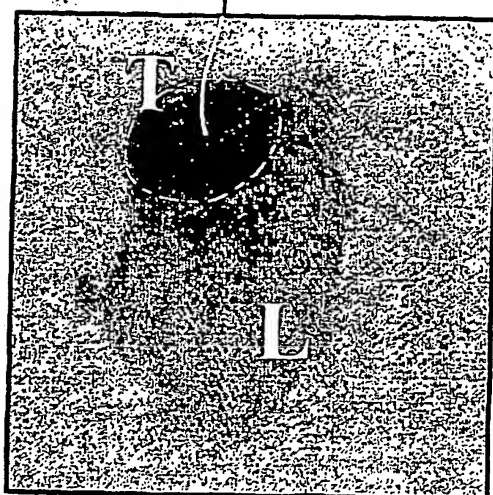


Control



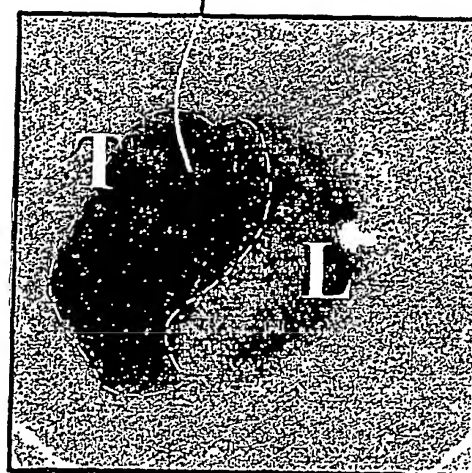
Ad-CMV-nLacZ

$\beta$ -gal positive



CTP1-nLacZ

$\beta$ -gal positive



CTP3-nLacZ

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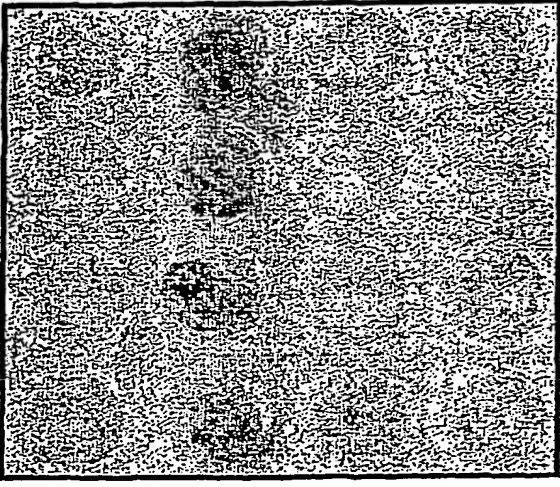
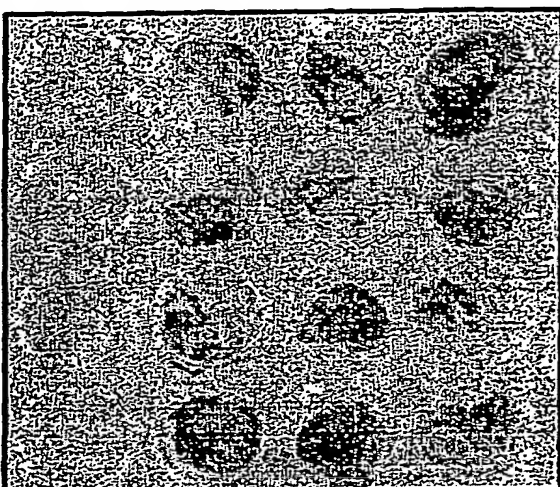


<p><b>A</b></p> <p>Control</p> <p>Ad-CMV-nLacZ</p> <p>CTP1-nLacZ</p> <p>CTP3-nLacZ</p>			<p>Patient B</p>
<p><b>B</b></p> <p><math>\beta</math>-catenin expression</p>			

FIG. 18

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/00856

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/47 C12N15/12 C12N9/02 C12N15/53 C12N15/66  
 A61K31/70 A61K48/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 939 122 A (UNIV UTRECHT) 1 September 1999 (1999-09-01) page 3, line 10 -page 8, line 12	1-3,17, 18,21-27
Y	GREEN N K ET AL: "Sensitization of colorectal and pancreatic cancer cell lines to the prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) by retroviral transduction and expression of the E. coli nitroreductase gene" CANCER GENE THERAPY,US,NORWALK, CT, vol. 4, no. 4, 1 August 1997 (1997-08-01), pages 229-238, XP002092056 ISSN: 0929-1903 the whole document	1-3,17, 18,21-27

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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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Date of the actual completion of the international search

27 July 2001

Date of mailing of the international search report

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Mateo Rosell, A.M.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/00856

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Ca	y *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A		MCNEISH ET AL: "Virus directed enzyme prodrug therapy for ovarian and pancreatic cancer using retrovirally delivered E. coli nitroreductase and CB1954" GENE THERAPY, GB, MACMILLAN PRESS LTD., BASINGSTOKE, 1998, pages 1061-1069, XP000901446 ISSN: 0969-7128 the whole document	1-3, 21-27
A		FRIDLOS F ET AL: "GENE-DIRECTED ENZYME PRODRUG THERAPY: QUANTITATIVE BYSTANDER CYTOTOXICITY AND DNA DAMAGE INDUCED BY CB1954 IN CELLS EXPRESSING BACTERIAL NITROREDUCTASE" GENE THERAPY, GB, MACMILLAN PRESS LTD., BASINGSTOKE, vol. 5, no. 1, January 1998 (1998-01), pages 105-112, XP000783674 ISSN: 0969-7128 the whole document	1-3, 21-27
A		DRABEK D ET AL: "The expression of bacterial nitroreductase in transgenic mice results in specific cell killing by the prodrug CB1954" GENE THERAPY, GB, MACMILLAN PRESS LTD., BASINGSTOKE, vol. 4, no. 2, 1 February 1997 (1997-02-01), pages 93-100, XP002092057 ISSN: 0969-7128 the whole document	1-3, 21-27
A		WO 93 08288 A (CANCER RES CAMPAIGN TECH) 29 April 1993 (1993-04-29) page 2, line 8 -page 16, line 2 & EP 0 638 123 A 15 February 1995 (1995-02-15) cited in the application	2, 3, 23-27
A		WO 98 41631 A (UNIV UTRECHT ;UNIV JOHNS HOPKINS (US)) 24 September 1998 (1998-09-24) page 2, line 5 -page 5, line 6; examples 1-4	1, 6-11, 17, 18, 21-27

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/00856

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MORIN PJ ET AL: "Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC 'see comments!'"</p> <p>SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 275, no. 5307, 21 March 1997 (1997-03-21), pages 1787-1790, XP002088507</p> <p>ISSN: 0036-8075</p> <p>cited in the application</p> <p>the whole document</p>	1
A	<p>KORINEK V ET AL: "CONSTITUTIVE TRANSCRIPTIONAL ACTIVATION BY A BETA-CATENIN-TCF COMPLEX IN APC-/- COLON CARCINOMA"</p> <p>SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 275, 21 March 1997 (1997-03-21), pages 1784-1787, XP002070159</p> <p>ISSN: 0036-8075</p> <p>cited in the application</p> <p>the whole document</p>	1
P,X	<p>DJEHA A HAKIM ET AL: "Expression of Escherichia coli B nitroreductase in established human tumor xenografts in mice results in potent antitumoral and bystander effects upon systemic administration of the prodrug CB1954."</p> <p>CANCER GENE THERAPY, vol. 7, no. 5, May 2000 (2000-05), pages 721-731, XP002173302</p> <p>ISSN: 0929-1903</p> <p>the whole document</p>	1-3, 21-27
P,X	<p>WEEDON SARAH J ET AL: "Sensitisation of human carcinoma cells to the prodrug CB1954 by adenovirus vector-mediated expression of E. coli nitroreductase."</p> <p>INTERNATIONAL JOURNAL OF CANCER, vol. 86, no. 6, 15 July 2000 (2000-07-15), pages 848-854, XP001015211</p> <p>ISSN: 0020-7136</p> <p>the whole document</p>	1-3, 21-27

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/00856

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Ca y°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>BRUNORI MICHELE ET AL: "Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway." JOURNAL OF VIROLOGY, vol. 75, no. 6, March 2001 (2001-03), pages 2857-2865, XP001010581 ISSN: 0022-538X the whole document</p>	1,5, 21-27

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 01/00856

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0939122	A	01-09-1999	AU 2860199 A	15-09-1999
			WO 9943804 A	02-09-1999
WO 9308288	A	29-04-1993	AU 681337 B	28-08-1997
			AU 2776992 A	21-05-1993
			AU 3521597 A	19-02-1998
			CA 2122036 A	29-04-1993
			EP 0540263 A	05-05-1993
			EP 0638123 A	15-02-1995
			JP 7501692 T	23-02-1995
			US 5977065 A	02-11-1999
			US 5633158 A	27-05-1997
			US 5780585 A	14-07-1998
WO 9841631	A	24-09-1998	US 5851775 A	22-12-1998
			AU 6765898 A	12-10-1998
			EP 0972037 A	19-01-2000
			US 5998600 A	07-12-1999
			US 6140052 A	31-10-2000